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## Marine Biological Laboratory

WOODS HOLE, MASS.


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# BIOLOGICAL BULLETIN

## STUDIES ON THE INFLUENCE OF INANITION ON THE DEVELOPMENT AND THE DURATION OF LIFE IN INSECTS.

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WITH 3 TABLES.

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The experiments were performed on the caterpillars of the common moth *Lymantria dispar* L. Each experiment consisted of several separate broods. The caterpillars deriving from one brood were divided in two parts and put in two jars, one part was subjected to starvation, the other, daily fed, was used as a control. The inanition applied in these studies was total but intermittent, the caterpillars being totally deprived of food during certain days and fed *ad libitum* during the remaining. The distribution of these days was different in different series of experiments, as may easily be seen from tables 1 to 3. The obtained pupæ being kept each separately, it was possible to study the influence of starvation not only on the duration of the stage of caterpillars, but also on that of each chrysalis and each moth.

In the chief experiments (series *A* to *F*) the whole material consisted of 1239 starved caterpillars, and 843 specimens for control; 547 pupæ were obtained from the caterpillars subjected to starvation and 763 from control larvæ; they produced 476 "starved" and 683 control moths (Cf. Table I.). The increased

[1] Paper from the Laboratory of Experimental Morphology., cf. *Mém. de l'Institut Nat. Polonais d'Économie Rurale à Pulawy*, Vol. 1, 1921.

## TABLE I

| Onset of Experiments. | Starvation Applied to: | Number Referring to Each Brood. | Number of Pupæ. | | | | Number of Moths. | | | | Weight of Pupæ in Mg. | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Starved. | | Control. | | Starved. | | Control. | | ♂♂. | | | | ♀♀. | | | |
| | | | | | | | | | | | Starved. | | Control. | | Starved. | | Control. | |
| | | | ♂♂. | ♀♀. | ♂♂. ♀♀. | | ♂♂. | ♀♀. | ♂♂. | ♀♀. | Limits of Individual Fluctuations. | Average. | Limits of Individual Fluctuations. | Average. | Limits of Individual Fluctuations. | Average. | Limits of Individual Fluctuations. | Average. |
| Since hatching from eggs. | *Series A.* Starvation lasting one day every second day (+ − + − + −) applied during the whole larval life. | 1 | 10 | 14 | 21 | 35 | 7 | 11 | 18 | 28 | 180–394 | 252.7 | 262–524 | 383.7 | 433 - 935 | 686.2 | 697–1277 | 1006.3 |
| | | 3 | 9 | 12 | 16 | 15 | 9 | 11 | 15 | 15 | 302–421 | 364.0 | 410–565 | 507.1 | 629–1158 | 963.0 | 872–1570 | 1261.6 |
| | | 4 | 15 | 16 | 15 | 30 | 12 | 14 | 12 | 24 | 215–480 | 304.6 | 180–471 | 376.8 | 612–1181 | 907.1 | 420–1264 | 870.6 |
| | | 11 | 10 | 13 | 35 | 38 | 10 | 12 | 30 | 31 | 225–317 | 271.3 | 184–572 | 430.5 | 348 - 852 | 698.5 | 485–1605 | 1057.4 |
| | *Series B.* The same starvation applied only during the first 20 days of larval life. | 2 | 10 | 14 | 14 | 24 | 8 | 11 | 12 | 22 | 222–433 | 345.3 | 271–471 | 394.4 | 755–1382 | 1087.7 | 611–1535 | 1175.0 |
| | | 9 | 12 | 8 | 15 | 16 | 12 | 8 | 12 | 11 | 395–565 | 439.6 | 280–556 | 388.0 | 470–1557 | 1097.2 | 538–1354 | 1030.8 |
| | | 12 | 10 | 10 | 17 | 16 | 8 | 10 | 14 | 10 | 291–502 | 416.2 | 275–514 | 399.3 | 563–1504 | 1148.8 | 598–1521 | 1073.1 |
| Since the last moult but one. | *Series C.* Starvation lasting one day every third day (+ + − + + −) | 34 | 15 | 9 | 12 | 9 | 14 | 8 | 12 | 9 | 340–425 | 366.8 | 468–533 | 496.1 | 760–1015 | 876.0 | 1257–1465 | 1341.6 |
| | | 35 | 18 | 11 | 18 | 8 | 14 | 10 | 18 | 7 | 273–395 | 333.7 | 447–586 | 509.1 | 414- 793 | 577.8 | 624–1587 | 1273.5 |
| | | 36 | 16 | 10 | 16 | 12 | 10 | 10 | 14 | 12 | 232–355 | 314.2 | 344–511 | 444.2 | 322–1280 | 833.6 | 996–1081 | 1034.5 |
| | | 37 | 12 | 12 | 16 | 10 | 12 | 12 | 14 | 10 | 247–372 | 303.3 | 335–507 | 409.0 | 620 - 917 | 814.2 | 820–1230 | 1042.6 |
| | | 38 | 15 | 18 | 16 | 16 | 12 | 14 | 16 | 16 | 255–369 | 306.6 | 419–557 | 466.2 | 444 1063 | 733.6 | 850–1284 | 1074.8 |
| | | 39 | 9 | 9 | 11 | 15 | 7 | 8 | 9 | 12 | 200–396 | 291.7 | 373–537 | 447.7 | 375– 739 | 581.1 | 734–1576 | 1131.8 |
| | *Series D.* Starvation lasting one day every second day (+ − + − + −) | 28 | 11 | 8 | 13 | 9 | 9 | 8 | 12 | 8 | 163–274 | 212.2 | 298–454 | 399.1 | 530 - 782 | 665.8 | 962–1293 | 1146.3 |
| | | 29 | 13 | 13 | 15 | 15 | 13 | 12 | 15 | 12 | 171–212 | 191.5 | 431–490 | 452.4 | 363– 590 | 475.0 | 745–1320 | 1115.0 |
| | | 30 | 16 | 9 | 15 | 10 | 16 | 7 | 15 | 10 | 185–302 | 242.5 | 401–567 | 445.4 | 290– 638 | 469.3 | 867–1285 | 1052.4 |
| | | 31 | 12 | 12 | 14 | 14 | 12 | 10 | 13 | 14 | 185–279 | 232.0 | 409–530 | 477.4 | 382 - 963 | 595.7 | 1147–1565 | 1329.4 |
| | | 32 | 12 | 12 | 12 | 13 | 12 | 9 | 12 | 12 | 145–298 | 230.1 | 279–500 | 419.5 | 373–1085 | 689.6 | 663–1352 | 1176.9 |
| | | 33 | 12 | 10 | 10 | 10 | 9 | 8 | 9 | 9 | 155–195 | 168.6 | 343–537 | 439.1 | 347 - 695 | 489.4 | 808–1350 | 1150.3 |
| | *Series E.* Starvation lasting two days every second day (+ − − + − −) | 40 | 6 | 8 | 7 | 14 | 6 | 6 | 6 | 14 | 121–207 | 154.0 | 422–552 | 480.2 | 156– 283 | 230.8 | 715–1371 | 1138.0 |
| | | 42 | 4 | 6 | 10 | 11 | 3 | 6 | 10 | 10 | 118–168 | 135.6 | 391–427 | 411.6 | 131– 378 | 269.1 | 705–1302 | 1059.3 |
| | | 45 | 6 | 6 | 13 | 10 | 6 | 4 | 13 | 10 | 100–131 | 112.5 | 355–540 | 446.8 | 172– 421 | 318.2 | 844–1355 | 1088.3 |
| | *Series F.* Starvation lasting two days every third day (+ + − − + + − −) | 46 | 10 | 8 | 14 | 16 | 6 | 7 | 14 | 14 | 165–272 | 173.7 | 365–525 | 443.4 | 299– 590 | 428.1 | 1112–1860 | 1340.5 |
| | | 47 | 8 | 10 | 8 | 16 | 8 | 10 | 6 | 14 | 158–210 | 182.5 | 445–602 | 516.2 | 355– 486 | 418.0 | 922–1528 | 1130.7 |
| | | 48 | 8 | 10 | 14 | 14 | 7 | 8 | 14 | 14 | 155–257 | 172.8 | 330–556 | 505.7 | 289– 473 | 395.2 | 913–1052 | 998.6 |

mortality of the starved caterpillars ought not to be ascribed first of all to the emaciating influence of inanition, the chief cause of this mortality is the moult. Directly after moulting the animal takes very much food, as during these processes it does not eat at all and only digests its own substances. Therefore when the fasting day falls on the period succeeding moult the caterpillars are often unable to resist starvation. The mortality of chrysalids, on the contrary, was identical in starved and in control specimens. The willow leaves on which the caterpillars were fed were always of the same variety and of the same freshness. The conditions of space, light and moisture were identical in all jars during the whole time of observations and the temperature was from 16° to 19° C. Food was put in great superfluity into control jars every morning and into those containing starved material during the feeding days and it preserved its freshness the whole day. All experiments were performed during one season (1920).

### 1. The Influence of Inanition on the Duration of Separate Developmental Stages.

For the experiments of series *A* caterpillars belonging to four different broods were used, from 1 to 6 hours after hatching. These caterpillars were deprived of food every second day, in the remaining days they were fed *ad libitum*. Starvation lasting one day and applied every second day may be designated by the symbol + − + − + − . Table II. records the limits of individual fluctuations in the duration of life of the caterpillars and chrysalids belonging to each brood, as well as the average duration of life in starved and in control specimens of the two sexes. Table III. finally shows the average differences of the duration of larval and of pupal life of individuals deprived of food for each brood separately, calculated in percentages of the average duration of the larval or of the pupal stages of control specimens of the same brood and the same sex. From these tables we see that the life of caterpillars subjected to such starvation was considerably prolonged. In all lots without exception the longest-lived control caterpillars underwent pupation earlier than the shortest-lived starved specimens of the same brood and sex. (Cf. Table II. *Series A*). If we take the average of the averages of all broods of series *A*, a quantity which I shall call "average of broods,"

TABLE II

| Onset of Experiments. | Starvation Applied to: | Number Referring to Each Brood. | Number of Days of Larval Life from Onset of Experiment till Pupation. | | | | | | | | Number of Days of Pupal Life. | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | ♂♂. | | | | ♀♀. | | | | ♂♂. | | | | ♀♀. | | | |
| | | | Starved. | | Control. | | Starved. | | Control. | | Starved. | | Control. | | Starved. | | Control. | |
| | | | Limits of Individual Fluctuations. | Average. | Limits of Individual Fluctuations. | Average. | Limits of Individual Fluctuations. | Average. | Limits of Individual Fluctuations. | Average. | Limits of Individual Fluctuations. | Average. | Limits of Individual Fluctuations. | Average. | Limits of Individual Fluctuations. | Average. | Limits of Individual Fluctuations. | Average. |
| Since hatching from eggs. | *Series A.* Starvation lasting one day every second day (+ − + − + −) applied to during the whole larval life. | 1 | 62–80 | 70.7 | 42–49 | 43.8 | 75–81 | 78.0 | 43–58 | 46.0 | 11–16 | 14.7 | 19–23 | 22.2 | 10–12 | 11.0 | 17–21 | 19.8 |
| | | 3 | 63–74 | 67.6 | 42–47 | 45.0 | 75–81 | 78.5 | 45–55 | 48.0 | 15–18 | 16.6 | 22–25 | 23.4 | 10–12 | 11.0 | 20–22 | 20.8 |
| | | 4 | 63–84 | 71.8 | 44–49 | 45.7 | 71–81 | 76.5 | 44–68 | 49.2 | 12–20 | 16.0 | 22–25 | 23.8 | 10–13 | 11.2 | 17–21 | 20.2 |
| | | 11 | 62–67 | 64.5 | 43–49 | 45.3 | 75–78 | 76.0 | 44–59 | 48.2 | 14–19 | 16.5 | 21–26 | 23.2 | 11–12 | 11.6 | 19–22 | 20.0 |
| | *Series B.* The same starvation applied only during the first 20 days of larval life. | 2 | 56–70 | 61.1 | 43–55 | 45.8 | 60–67 | 63.0 | 44–59 | 48.6 | 16–21 | 19.2 | 21–25 | 23.7 | 15–17 | 16.0 | 18–22 | 20.4 |
| | | 9 | 57–69 | 62.5 | 44–68 | 49.4 | 63–72 | 68.3 | 45–68 | 52.5 | 17–21 | 19.0 | 18–24 | 23.0 | 13–17 | 15.3 | 16–21 | 20.1 |
| | | 12 | 63–77 | 69.3 | 44–69 | 54.4 | 64–71 | 67.6 | 44–64 | 51.6 | 14–20 | 17.8 | 16–24 | 21.2 | 14–18 | 16.0 | 18–22 | 20.6 |
| Since the last moult but one. | *Series C.* Starvation lasting one day every third day (+ + − + + −) | 34 | 21–23 | 22.2 | 18–20 | 18.8 | 23–37 | 28.1 | 19–20 | 19.6 | 22–23 | 22.2 | 22–24 | 23.1 | 16–23 | 19.8 | 20–22 | 21.0 |
| | | 35 | 21–24 | 22.4 | 18–20 | 18.8 | 26–36 | 30.0 | 20–27 | 21.5 | 21–24 | 22.5 | 22–24 | 23.2 | 16–23 | 19.8 | 20–22 | 21.0 |
| | | 36 | 20–28 | 22.5 | 18–21 | 19.6 | 24–36 | 29.4 | 20–22 | 20.6 | 21–22 | 21.6 | 21–23 | 22.2 | 16–21 | 19.0 | 19–22 | 20.1 |
| | | 37 | 21–28 | 23.3 | 19–21 | 19.7 | 23–31 | 26.2 | 20–21 | 20.4 | 20–23 | 21.5 | 20–25 | 22.4 | 18–20 | 19.2 | 20–21 | 20.6 |
| | | 38 | 20–22 | 21.4 | 18–22 | 19.5 | 22–28 | 23.8 | 20–22 | 20.6 | 22–24 | 23.2 | 23–25 | 24.0 | 19–20 | 19.7 | 20–22 | 20.7 |
| | | 39 | 21–32 | 23.3 | 19–20 | 19.8 | 22–35 | 26.3 | 19–20 | 21.1 | 21–23 | 22.1 | 21–24 | 22.8 | 18–20 | 19.3 | 19–21 | 20.2 |
| | *Series D.* Starvation lasting one day every second day (+ − + − + −) | 28 | 24–30 | 27.0 | 16–22 | 18.0 | 26–29 | 26.8 | 17–21 | 19.2 | 20–22 | 21.3 | 22–23 | 22.6 | 17–22 | 19.8 | 20–22 | 21.0 |
| | | 29 | 25–27 | 26.0 | 16–19 | 17.8 | 26–30 | 28.0 | 18–22 | 19.4 | 21–23 | 22.0 | 22–24 | 22.8 | 18–20 | 19.3 | 20–21 | 20.7 |
| | | 30 | 23–25 | 24.0 | 17–19 | 18.0 | 23–39 | 29.5 | 16–20 | 18.2 | 21–22 | 21.5 | 23–25 | 23.8 | 16–20 | 18.4 | 20–21 | 20.6 |
| | | 31 | 24–25 | 24.5 | 17–20 | 18.5 | 23–39 | 28.8 | 18–23 | 20.5 | 21–23 | 22.0 | 21–24 | 23.0 | 19–22 | 20.5 | 21–23 | 21.7 |
| | | 32 | 23–33 | 26.3 | 17–22 | 19.1 | 23–38 | 30.2 | 18–25 | 20.1 | 20–24 | 22.0 | 21–24 | 22.9 | 16–21 | 19.0 | 19–22 | 20.3 |
| | | 33 | 24–33 | 29.0 | 18–22 | 20.1 | 27–43 | 35.1 | 20–23 | 21.2 | 21–22 | 21.6 | 22–24 | 23.2 | 14–20 | 17.3 | 20–23 | 21.0 |
| | *Series E.* Starvation lasting two days every second day (+ − − + − −) | 40 | 27–32 | 29.3 | 18–19 | 18.4 | 30–40 | 37.0 | 18–23 | 19.5 | 16–21 | 18.6 | 22–24 | 22.8 | 13–15 | 14.3 | 20–22 | 21.0 |
| | | 42 | 25–38 | 31.2 | 18–20 | 18.8 | 28–45 | 37.0 | 18–22 | 19.3 | 17–19 | 18.0 | 22–23 | 22.4 | 12–15 | 13.6 | 19–21 | 19.7 |
| | | 45 | 26–35 | 31.3 | 17–21 | 18.3 | 28–48 | 37.1 | 19–21 | 19.8 | 16–19 | 17.3 | 22–25 | 23.8 | 13–16 | 14.7 | 19–21 | 20.3 |
| | *Series F.* Starvation lasting two days every third day (+ + − − + + − −) | 46 | 22–38 | 28.9 | 18–20 | 18.7 | 33–39 | 36.5 | 18–24 | 19.8 | 17–21 | 19.8 | 21–23 | 22.4 | 14–16 | 15.1 | 19–22 | 20.2 |
| | | 47 | 32–34 | 33.0 | 19–24 | 20.8 | 37–39 | 38.0 | 20–24 | 21.7 | 18–20 | 19.0 | 23–24 | 23.3 | 13–16 | 14.5 | 20–21 | 20.4 |
| | | 48 | 21–39 | 27.1 | 18–19 | 18.1 | 29–37 | 33.2 | 18–20 | 19.0 | 17–21 | 19.4 | 23–25 | 23.8 | 15–18 | 16.5 | 20–21 | 20.6 |

## TABLE III

| | | Experiment Begun Since Hatching from Eggs. | | | | | | | | Experiment Begun Since the Last Moult But One. | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *Series A* Starvation Lasting One Day Every Second Day (+ − + − + −) Applied During the Whole Larval life. | | | | | *Series B.* The Same Starvation Applied to Only During the First 20 Days of Larval Life. | | | *Series C.* Starvation Lasting One Day Every Third Day (− ‡ − ‡ ‡ −). | | | | | | | *Series D.* Starvation Lasting One Day Every Second Day (+ − + − + −). | | | | | | | *Series E.* Starvation Lasting Two Days Every Second Day (+ − − + − −). | | | | *Series F.* Starvation Lasting Two Days Every Third Day (+ + − − + + − −). | | | |
| | | Number Referring to Each Brood. | | | | Average of Broods. | Number Referring to Each Brood. | | | Number Referring to Each Brood. | | | | | | Average of Broods. | Number Referring to Each Brood. | | | | | | Average of Broods. | Number Referring to Each Brood. | | | Average of Broods. | Number Referring to Each Brood. | | | Average of Broods. |
| | | 1 | 3 | 4 | 11 | | 2 | 9 | 12 | 34 | 35 | 36 | 37 | 38 | 39 | | 28 | 29 | 30 | 31 | 32 | 33 | | 40 | 42 | 45 | | 46 | 47 | 48 | |
| Average prolongation of life of the starved caterpillars in per cent. of the average duration of larval life of control specimens of the same brood and sex, from the time of onset of experiment till pupation. | ♂ | 61.4 | 50.2 | 57.1 | 42.3 | 52.7 | 33.4 | 26.5 | 27.3 | 18.0 | 19.1 | 14.7 | 18.2 | 9.7 | 17.6 | 16.— | 50.0 | 40.0 | 33.3 | 32.4 | 37.6 | 14.2 | 10.5 | 59.2 | 65.9 | 71.0 | 65.3 | 54.5 | 58.6 | 49.7 | 54.2 |
| | ♀ | 69.5 | 63.5 | 55.4 | 57.6 | 61.5 | 29.2 | 30.0 | 31.0 | 30.0 | 43.3 | 39.5 | 42.7 | 28.4 | 15.5 | 24.6 | 32.3 | 30.5 | 14.3 | 62.9 | 19.4 | 50.2 | 65.5 | 50.3 | 89.7 | 91.7 | 87.3 | 89.5 | 81.3 | 75.1 | 74.7 | 78.0 |
| Average abbreviation of life of the "starved" pupæ, in per cent. of the average duration of pupal life in control specimens of the same brood and set. | ♂ | 33.7 | 29.0 | 32.7 | 28.8 | 31.0 | 18.9 | 17.3 | 16.0 | 17.4 | 3.8 | 3.0 | 2.7 | 4.0 | 3.3 | 3.0 | 3.3 | 5.7 | 3.5 | 9.6 | 4.3 | 3.9 | 6.8 | 5.6 | 18.4 | 19.6 | 27.3 | 21.7 | 11.6 | 18.4 | 18.4 | 16.1 |
| | ♀ | 44.4 | 47.1 | 44.5 | 42.0 | 44.5 | 21.5 | 23.8 | 22.3 | 22.5 | 5.7 | 5.7 | 5.0 | 6.7 | 4.8 | 4.4 | 5.3 | 5.7 | 6.7 | 10.6 | 5.5 | 6.4 | 17.6 | 8.7 | 31.9 | 30.9 | 27.5 | 30.1 | 25.2 | 28.8 | 19.9 | 24.6 |
| Average weight of the "starved" pupæ in per cent. of the average weight of control chrysalids of the same brood and set. | ♂ | 95.5 | 71.7 | 80.9 | 63.0 | 70.2 | 87.5 | 113.2 | 104.2 | 101.6 | 73.9 | 65.5 | 70.7 | 74.1 | 65.7 | 65.1 | 69.1 | 53.1 | 42.3 | 54.4 | 48.5 | 54.8 | 38.3 | 48.5 | 32.0 | 32.9 | 25.1 | 30.0 | 39.1 | 35.3 | 34.1 | 36.1 |
| | ♀ | 68.1 | 76.3 | 104.1 | 66.0 | 78.3 | 92.5 | 106.4 | 107.0 | 101.9 | 65.2 | 45.3 | 80.5 | 78.0 | 68.2 | 51.3 | 64.7 | 58.0 | 42.6 | 44.5 | 44.8 | 58.5 | 42.5 | 48.4 | 20.2 | 25.4 | 29.2 | 24.9 | 31.7 | 36.9 | 39.5 | 36.0 |

we obtain for male specimens a prolongation of larval life of 52.7 per cent. for the female of 61.5 per cent. Cf. Table III. As the processes of pupation were checked every 24 hours and the caterpillars were taken for the experiments from 1 to 6 hours after their hatching from eggs, the error concerning duration of the larval stage could have in no case exceeded 30 hours.

The duration of the pupal stage is influenced by the starvation of caterpillars in an essentially different manner, viz, the chrysalids which have developed from starved caterpillars undergo transformation into adult moths far earlier than control pupæ, but the abbreviation of the pupal stages is smaller than the prolongation of larval life. This abbreviation amounted in the "averages of broods" in males to 31.0 per cent. in females to 44.5 per cent. of the average duration of pupal stage of control specimens. Pupation having been controlled every 24 hours, emergence of moths every 12 hours, the error in estimation of the duration of the pupal stage could not exceed 36 hours.

The moths of *Lymantria dispar* L. do not take any food in their imaginal stage. Control males lived as moths in separate lots from 1 to 8, from 5 to 8, from 2 to 8 and from 1 to 8 days and the "starved" specimens from 4 to 5, from 5 to 11, from 2 to 8 and from 1 to 6 days. Normal females lived from 3 to 13, from 1 to 10, from 1 to 12 and from 2 to 14 days, those derived from starved caterpillars from 5 to 6, from 3 to 10, from 7 to 10 and from 6 to 10 days. Comparing the mean values of the duration of life of the normal and of the "starved" moths we obtain in most cases a certain prolongation of life of the moths derived from starved caterpillars (+) though rarely a certain abbreviation (−). This prolongation or abbreviation calculated in days amounts in separate broods in the males to + 0.3, + 2.5, + 0.1 and −0.1 days, in the females to −2.4, + 0.3, + 2.8 and + 2.1 days. We see that starvation of caterpillars has no distinct effect on the duration of the imaginal stage. Emergence and death of moths having been checked every 12 hours, the error in the estimation may attain only 24 hours. The moths of the two sexes experimented upon both control and "starved" have never been allowed to mate.

If we take into consideration the behavior of the "starved" moths as well as the fact that the abbreviation of the pupal

period was smaller than the prolongation of the larval stage, we must draw the conclusion that total deprivation of food of caterpillars every second day has a considerable positive effect on the total duration of their life from hatching until death. The prolongation of the whole developmental period amounts here in the average of broods in males to 16.5 and in females to 20.4 days, or in percentages relative to the duration of life of control specimens in males to 24.2 per cent. and in females to 30.0 per cent.

The above results find complete confirmation in my farther comparative experiments in which inanition of various intensity was applied (series *C*, *D* and *E*). In these experiments caterpillars were used from 1 to 12 hours after their last moult but one. In series *C* food was administered two days, the third day the insects were starved (starvation lasting one day every third day + + − + + − + + −). In series *D* the animals were again deprived of food every second day (starvation lasting one day every second day + − + − + −) and the caterpillars of series *E* were deprived of food two days and ate only every third day (starvation lasting two days every third day + − − + − − + − −). From the absolute figures of Table II, as well as from the percentages in Table III we may infer that the duration of the larval period undergoes also, in these starved animals considerable prolongation (cf. analogous results obtained in silk-worms by Kellogg and Bell, '04 *b*), simultaneously with abbreviation of the pupal stage. These changes become more and more remarkable in proportion as inanition increases, they are larger in series *D* than in series *C* and the largest in series *E* in which the fasting days were the most numerous. Also in these comparative experiments the prolongation of the larval life was greater than the abbreviation of the pupal period, while the duration of the imaginal life remained unchanged. Hence it follows that the total duration of the life of the insects from the beginning of the experiment till death of the moth, is more and more increased in proportion as inanition increases within the limits of the experiments. The duration of development from the last moult but one till the emergence of the moth underwent in the average of broods a prolongation (calculated in percentages of the average duration of such control periods) which in the males

amounted to 5.6 per cent. in series *C*, to 15.1 per cent. in series *D* and to 16.8 per cent. in series *E*; in the females the prolongation was 13.3 per cent. in series *C*, 20.2 per cent. in series *D* and 28.6 per cent. in series *E*.

In series *F* the caterpillars were fed during two days and deprived of food during the next two. (Starvation lasting two days every third day $++--++--$). Notwithstanding a different distribution of the feeding and fasting days, the quantitative relation of these days was the same as in series *D* to which starvation lasting one day every second day was applied ($+-+-+-$); in both experiments the caterpillars experimented upon were of the same age. It nevertheless turned out that the prolongation of life was much greater in caterpillars of series *F* than in specimens belonging to series *D*. (Cf. Table II. and III.) In series *F* it amounted in the average of broods in the males to 54.2 per cent. of the average of the life of control caterpillars from their last moult but one, and in the females to 78.0 per cent while in series *D* the corresponding numbers were 40.5 per cent. in males and 50.3 per cent. in females. The abbreviation of the pupal period calculated similarly was in series *F*, in males 16.1 per cent of the duration of life of control chrysalids and in females 24.6 per cent. while in series *D* the life of male pupæ underwent abbreviation of 5.6 per cent. and of the female 8.7 per cent. We see that changes of the duration of the larval and of the pupal stages induced by inanition depend not only on the mutual quantitative relation of the fasting and feeding period, but also on the distribution of these periods. The organism responds by more energetic reaction to longer, though less frequent, fasting intervals than to more frequent but shorter periods of starvation.

### 3. Inanition and its Bearing on the Physiology of Insect Metamorphosis.

When we try to discuss the foregoing results on the basis of hitherto existing references in the literature, we meet certain discrepancies which, however, may be cleared up by consideration of the physiology of animal metamorphosis.

Kellner ('87) fed caterpillars of the silk-worm during their whole life on insufficient quantities of leaves, and obtained a

short prolongation of the larval stage. Analogous experiments were afterwards made by Kellogg and Bell ('04 *a* and *b*). Pictet ('05) came to the conviction that the larval stage is prolonged and the pupal stage shortened by feeding caterpillars on plants containing few food-stuffs, but that the conjoint periods of development do not undergo any changes. A prolongation of larval life caused by inadequate food was lately determined by Northrop ('17) Tangl. ('09*a*), finally, maintains that the larvæ of flies reared on pure egg-white which they refused to take underwent transformation approximately a week later than normally. These results are, in general, concordant with my observations on starved caterpillars. On the other hand, the observations of Krizenecky ('14) and of Szwajsówna ('16) on the larvæ of *Tenebrio molitor* which underwent metamorphosis earlier when totally starved contradict the above determinations. A similar discrepancy may also be remarked in analogous investigations on amphibians. While Barfurth (87) and others have ascertained that starvation of amphibians causes acceleration of their metamorphosis, other investigators induced retardation of these processes by the administration of poor food to tadpoles. It has been emphasized by Wolterstorff ('96), Morgan ('07), Kaufman ('18) and others that in amphibians the influence probably differs in different developmental stages of the tadpoles used for starvation. The former of the above mentioned investigators deprived large tadpoles of food, in part shortly before their transformation, while the others used far younger specimens. Similarly the experiments of Kellner and the following authors and also my own were made on young caterpillars while the observations of Krizenecky and of Szwajsówna refer to older larvæ. In another experimental study on starved tadpoles (Kopeć, *22a* and *b*) I succeeded in confirming the above views of Wolterstorff, Morgan and others. My experiments showed that metamorphosis of the tadpoles to which starvation was applied before the fiftieth day of their development undergoes retardation, where as transformation is accelerated when tadpoles are starved after the sixty fifth day of their life. In this study on caterpillars similar behavior was also noted in one and the same material of female larvæ. It turned out that the caterpillars to which total starvation has been applied approximately since

the seventh day after their last moult underwent pupation in three different broods after from 7 to 13, from 7 to 16 and from 7 to 15 days (or on the average after 10.6, 10.8 and 10.0 days), whereas the control specimens of these broods become chrysalids as early as after from 6 to 12, from 6 to 12 and from 5 to 12 days (or on the average after 8.2, 9.3 and 8.6 days). On the contrary the larval life of the females of the same broods in which starvation began approximately from the tenth day after the last moult was much shorter than that of control larvæ, as pupation occurred here from 4 to 8, from 5 to 8 and from 4 to 8 days (or on the average after 5.5, 6.6 and 6.8 days), in control specimens from 5 to 10, from 6 to 10 and from 7 to 9 days (or on the average after 7.4, 7.6 and 8.1 days). Hence follows: (1) The character of the influence, whether accelerating or retarding, exerted by starvation on the processes of metamorphosis depends on the period of life or the developmental stage on which the factor begins. (2) This moment may be accurately determined by means of experiment, viz, in the females of *Lymantria dispar* L. In my cultures the critical moment during which the influence of inanition delaying metamorphosis is changed into an accelerating one fell on the period between approximately the seventh and the tenth day after the last moult.

An essential explanation of such different behavior of caterpillars may be found in my former studies on the importance of the brain for insect metamorphosis (Kopeć, '17 and '22*c*). It was shown that the female caterpillars of this moth, deprived of brain the seventh day after their last moult, live far longer than control animals, but they die without undergoing transformation. On the other hand, the removal of the brain from caterpillars the tenth day after their last moult has no influence either on the processes of pupation or on the emergence of moths. From various experiments supported by observation on control material it follows that the brain plays here most probably the rôle of an organ of internal secretion. From these results we may infer that by depriving caterpillars of food since the tenth day after their last moult we afford exceptionally favorable conditions for metamorphosis, viz, the substance or substances already produced by the brain in sufficient quantities find in such starved organisms less material which ought to be transformed

in time. The prolongation of the larval period in younger caterpillars starved before the seventh day after the last moult may be explained, on the contrary, by impeded secretoric function of the brain or by certain anomalies of its activity in starved organisms.

If the processes occurring in the pupa were a continuation of those taking place in the caterpillar and leading to pupation, the abbreviation of the pupal stage in starved specimens could not be reconciled with the above explanation. But these processes differ not only energetically as it has been shown by Tangl ('09*b*). The processes of pupation may morphologically be reduced to the histolysis of almost all larval tissues into a homogeneous mass which fills up the pupal body, whereas the transformation of the pupa into the fully-developed insect consists in the developemnt of the so-called imaginal discs, *i.e.*, of small accumulations of cells. In my present experiments (series *B*) I succeeded moreover in establishing certain physiological characters which point to another difference. In series *B* the caterpillars were deprived of food every second day for 20 days after their hatching from eggs, *i.e.*, at a time when there are not even any traces of histolytic processes in the larval body. From the twenty-first day till the end of the larval period the animals were fed every day and underwent the normal number of moults. From the appertaining items on Tables II. and III. we see that the caterpillars belonging to this series not only remarkably delayed the term of their pupation, but also that the duration of the pupal stage was considerably shortened. This abbreviation amounted in the average of broods to 17.4 per cent. of the average duration of life of normal pupae in males and to 22.5 per cent. in females. Consequently, the evolutive processes characteristic of the pupa are not a continuation of those changes which are characterized by histolysis of larval tissues. We see that by temporarily starving young caterpillars (Series *B*) we may segregate these processes and prove that processes of the development of the imaginal discs may begin far earlier, *i.e.*, soon after the hatching of the caterpillar from the egg. It ought therefore to be inferred that the brain has two separate functions in normal conditions, (1) it causes histolysis of larval tissues, (2) it delays the evolution of imaginal discs. During inanition of caterpillars the influence of the brain hindering the development of imaginal discs

is decreased, owing to its lowered function. The starved chrysalids therefore begin their pupal stage when the discs are better developed and this causes acceleration of the development of the imaginal body and hence abbreviation of the pupal life.

The assumption that the larval brain exerts two different influences is neither astonishing nor incomprehensible in respect to the well-known data concerning the physiology of organs of internal secretion. The final development of the imaginal discs and their definitive differentiation sets in before pupation or even in the pupa, *i.e.*, at a time when almost all larval tissues are undergoing histolysis or have undergone it. Therefore it ought to be admitted that during this period the influence of the brain hindering the development of embryonal discs normally becomes annuled by certain processes which are unknown to us. It is very probable that the development of imaginal discs and the histolysis of the larval tissues become, at least towards the end of larval life, physiologically correlated with each other. In the contrary case it could be hardly understood why the caterpillars deprived of their brain the seventh day after their last moult do not, it is true, exhibit any histolytical changes in their tissues, but the imaginal discs contained in their body do not undergo final growth and differentiation. (Kopeć, '17 and '22 *c*). Indeed, if there were no such correlation, the imaginal discs of the caterpillars deprived in that period of their brain (and therefore of the organ retarding their evolution) ought to develop the organs of the imago in spite of the absence of histolytical processes in the larval body.

In contrast to my experiments on the starvation of caterpillars, Loeb and Northrop ('17) have lately convinced themselves that each of the separate stages of life in Drosophila occurs more slowly in lower temperature and more quickly in higher ones. The changes of temperature were applied by the mentioned authors during the whole development of the animals, while in my experiments the factor of inanition had a direct influence only on the processes taking place in caterpillars, as the chrysalis does not take food in normal conditions either. Loeb and Northrop obtained the same changes of the duration of the larval and of the pupal stage first of all owing to changed celerity of metabolism in the larva as well as in the pupa. Experiments in which

lower or higher temperatures should be applied only to larvae, the chrysalids being kept in normal conditions, might solve the problem as to whether and to what degree the changed temperature has an influence not only on the celerity of metabolism, but also on the supposed function of the brain.

The attempts hitherto made to explain the metamorphosis of insects refer to the last stage of the processes. The appearance of phagocytosis, of degeneration, of asphyxia and of other "causes" of transformation is not yet elucidated. My experiments on the function of the larval brain as well as the discovery of tyrosinase in caterpillars and chrysalids made by Dewitz ('05 and '16) and confirmed by Steche and Waentig ('13) together with the present results on the starvation of caterpillars may lead to a better knowledge of the cause of insect metamorphosis. But my "secretory" theory of metamorphosis will not be well grounded until we succeed in finding in the structure of the brain an adequate base for the theory, *i.e.*, until certain specific changes in the brain not only during pupation, but also during moults will have been ascertained. A great support might be afforded by positive experimental results on transplantation of brains, or on injections of extracts of this organ into brainless specimens. The latter experiments only would be able to dispel every doubt in regard to the validity of my previous conclusion that the indubitable influence of the brain on metamorphosis is due to an internal secretion of this organ.

Deegener ('09) lastly considers the imaginal form to be phylogenetically older than the larval form, which developed secondarily from the fully developed insect owing to numerous secondary life conditions "unter fortgehender Retardation der Entwicklung imaginaler Organe" (p. 11). I think that my experiments of series *B* distinctly prove that in normal circumstances the development of the imaginal discs is retarded during larval stages. I cannot deny that in the light of these results the supposed retardation of the development of the imaginal organs gains an experimental base, at least in ontogenetic evolution.

### 3. Inanition and Certain Problems of Growth.

In this chapter I take into account only the final stages of growth of caterpillars expressed by the weight of new-formed

pupæ. The chrysalids were weighed from 1 to 24 hours after pupation, control weighings having proved that the weight of pupæ decreased in my broods during this period only from 0.18 per cent. to 0.33 per cent. The limits of fluctuations in the weight of pupæ are recorded in Table I., together with their average values. On Table III. we see moreover the average weight of "starved" chrysalids of each brood calculated in percentages of the average weight of control chrysalids of the same sex and brood.

On comparing the data of series *C*, *D* and *E* we see that the weight of chrysalids decreases more and more in proportion as the number of fasting days increases. Assuming in general that the number of feeding days in separate series corresponds to the quantity of food which has been taken, we may say that the weight of pupæ is in direct relation to the quantity of food consumed. If we also take into consideration the data of series *F* in comparison to analogous items of series *D*, we draw the conclusion that the average decrease of chrysalids due to inanition of caterpillars is larger when the feeding intervals are longer though less frequent. As it was pointed out, the prolongation of the stage of caterpillar and the abbreviation of that of pupa increases in proportion as more and more intense starvation has been applied. Consequently, the weight of the pupæ the caterpillars of which had been starved is in inverse relation to the prolongation of the larval period as well as to the abbreviation of the stage of chrysalids. Adopting the ratio of the pupal weight to the duration of larval life as the approximative measure of the rate of growth, we can infer from Tables I. to III. that this rate decreases in proportion as more and more intense starvation has been applied and in relation to the distribution of the feeding and fasting days (Series *F* and *D*). The above-stated principles refer to separate series of experiments but not to separate specimens, either control or starved, in one brood. I have often observed that although the processes of transformation lasted in the control or in the starved material in every brood several days, the heaviest and the lightest caterpillars underwent pupation the same day, sometimes the first and sometimes not until the last day, although the one was two- or threefold heavier than the other. The same may be said as to the duration of pupal

life in regard to the weight of separate chrysalids of one brood.

The problem arises whether the capacity to grow is checked by age of the animal. Such limits in rats have been adopted by Aron ('12) and lately by Jackson and Stewart ('20), in contrast to Osborne and Mendel ('14) who are inclined to the opinion that the capacity to grow is exhausted by mere growth, without regard to the factor of time. In series *B* of my experiments the caterpillars, deprived of food intermittently for 20 days since their hatching, weighed the twenty-first day in separate broods on the average only 7.6, 3.6 and 5.0 mg., while control individuals of the same broods had the mean weight of as much as 36.0, 45.5 and 40.3 mg. The starved specimens fed daily since the twenty-first day attained and partly exceeded in time the weight and size of control caterpillars (cf. weights on Table I. and III.). As in this series the processes of metamorphosis of originally starved caterpillars were retarded and as, on the other hand, caterpillars have grown until the end of their larval life, it ought to be inferred that in these animals the capacity to grow is not suppressed at a period at which caterpillars normally cease to grow. Moreover, it may be concluded that pupation also may take place much beyond the age at which the control specimens undergo pupation. But the inference that the capacity may not at all be limited by the age of animals ought not to be drawn from such results. It is possible that the rats experimented upon by Mendel and Osborne as well as my caterpillars would lose the capacity to obtain the weight and size of control specimens if they had been kept at maintenance or starved even a few days longer.

### 4. Adaptation of Organisms to Starvation. Some Remarks on the Problem of Death.

The weight of pupæ from series *A* (in which starvation lasting one day every second day, + − + − + −, was applied to caterpillars during the whole larval life) is evidently different from that of series *D* in which older specimens after their last moult but one were subjected to the same starvation (cf. Table I.). We see that organisms may in time get accustomed to the detrimental effects of starvation, which prevent the animal from attaining its normal weight: the comparison of the limits of individual fluctuations shown on Table I. points to the conclusion

that the chrysalids of series *D* which have been deprived of food only during the half of their larval life are lighter than those from series *A* the caterpillars of which have been starved during their whole larval life. From Table III. we see that the male pupæ from series *D* weighed in the average of broods 48.5 per cent., the female pupæ 48.4 per cent. of the average weight of the control chrysalids, whilst the male chrysalids from series *A* weighed as much as 70.2 per cent., the females 78.3 per cent.

As the prolongation of the larval period was much greater in series *A* than in series *D* (cf. Table I.), it might be supposed that the caterpillars of series *A* are heavier owing to the circumstance that they lived longer, and therefore could take and digest food during a longer period. But the following argument contradicts this opinion. At the outset of the experiment in series *D* the caterpillars weighed in the average of broods 94.5 mg., the male specimens underwent pupation in the average of broods after 26.1, the female after 29.7 days. If we take as starting point for series *A* the day on which the caterpillars of this series had an analogous weight, which in the average of broods amounted to 93.5 mg., the duration of larval life in this series from this day till pupation was in the average of broods 27.6 in males and 36.2 in females. We see that when the starting point had been made uniform the duration of farther larval life in series *A*, especially in males, is almost identical with that in series *D*. In other words, the specimens of series *A* attain considerably greater weight than those of series *D* during approximately the same period. The ratio of the produced number of milligrams of body-weight to the total number of days of the period during which the larvæ had been deprived of food being considered as rate of growth, we may calculate that the rate of growth amounts in the average of broods in series *A* to 7.4 in starved males and to 19.8 in females, whilst in series *D* this quantity attains only 4.5 in starved males and 15.8 in females. It follows that the starved organism may in time get accustomed to the metabolism of inanition, *i.e.*, the ratio of assimilation to disassimilation becomes during long starvation changed in favor of the organism.

By histological research I have convinced myself that caterpillars which died from starvation contain no adipose tissue. In a certain contrast to my observations, Bialaszewicz ('19) draws

from his experiments on the starvation of leeches as well as from the papers by several authors on other animals the conclusion supported by numerous grounds that fat has no great importance in the hunger metabolism of cold-blooded animals, but that it undergoes only small reduction. The farther investigations of Bialaszewicz will undoubtedly show whether my supposition based on histological research is right, or they may explain the cause of this discrepancy which possibly consists in the physiological capacity of caterpillars to digest their store of adipose tissue during their frequent moults.

In contemporary research the cause of natural death is regarded as caused by the accumulation of detrimental products of normal metabolism which cannot be removed from the multicellular organism. This view is not at all proved; it is based first of all on the known investigations of Woodruff on the infusoria whose conclusions are in discrepancy with the results obtained by Viewegerowa and Vieweger ('22) who by methodically exact research proved that the products of metabolism have no great importance on the development of Colpidium and that the divisions are hindered in unchanged surroundings first of all by inanition. It nevertheless seems to me to be unquestionable that the duration of life depends on the character of metabolism, in other words, that natural death is a function of metabolism. The experiments performed by Kellogg and Bell ('04 *b*), by Pictet ('04) and by Northrop ('17) on larvæ as well as those by Loeb and Northrop ('17) on fully-developed insects of Drosophila show that by means of inadequate food we may elicit considerable changes of the duration of development or of life of these organisms. As investigations on the hunger metabolism evidence distinct differences of the digested substances and of the character of their disintegration in comparison to normal metabolism, it ought to be inferred that, if death is a function of metabolism, insufficient feeding may also influence the moment of natural death. Schultz ('05) emphasizes that certain animals undergoing periods of hibernal (or æstival) sleep which is connected with very restricted metabolism live very long in comparison with those having no such periods of rest. Stress must be laid on the fact that in my experiments moths deriving from starved caterpillars in which the development was much prolonged or

delayed are much older organisms than the control moths. As the imago of *Lymantria dispar* L. never takes any food, the quantity of provisions stored in its body decides the duration of this life-period. From histological research it follows that the amount of this provision which may be noticed in the imago (the so-called adipose body) is in the starved specimens, in relation to their decreased body, not at all smaller than in the controls. This is due to the fact that the starved caterpillars do not form normally sized chrysalids, which contain smaller quantities of the mentioned body, but they are transformed into smaller, even dwarfed specimens. By this circumstance it might be explained why both categories of moths live in general equally long. But in the case of the "starved" moths derived from caterpillars the life of which has undergone a very remarkable prolongation it is obvious that the hunger metabolism during development caused retardation of natural death of the organism. This I consider to point clearly to the conception of death as a function of the character of general metabolism. According to Ruzicka's researches ('17), the newts, when totally deprived of food, undergo moults more rapidly than control specimens. In connection with my experiments it would be very interesting to ascertain whether the duration of life of such newts undergoes changes.

More detailed considerations on the cause of especially favorable influence of intermittent starvation on duration of life of animals are to be found in my former papers (Kopeć, '22 *a* and *b*.)

## 5. Summary.

1. Intermittent starvation of young caterpillars of *Lymantria dispar* L. causes considerable prolongation of the larval life as well as a certain abbreviation of the pupal period but has no influence on the duration of life of the imago. These changes increase in proportion as more intense starvation is applied. Larger effects are elicited by longer and less frequent than by more frequent, but shorter, feeding intervals.

2. The differences of results obtained by various authors in regard to the influence of starvation on metamorphosis depend on differences of age of the animals experimented upon. The caterpillars subjected to inanition approximately from the seventh

day after their last moult had retarded pupation, whereas this process is accelerated by starvation of animals approximately since the tenth day after the last moult.

3. The development of imaginal discs is not the consequence of histolytical processes which cause pupation of caterpillars, but they take place simultaneously from the first days of larval life. The brain causes, probably by its secretion (or secretions), histolysis of larval tissues and it also seems to check in the caterpillar the development of the imaginal discs.

4. The prolongation of the larval period in starved specimens may be explained by certain disturbances in the hypothetical secretory function of the larval brain, which are caused by inanition; the abbreviation of the pupal stage may be ascribed to analogous decrease of the influence of this organ, which retards the development of imaginal discs.

5. The average limit of larval growth expressed in the average weight of the new-formed chrysalids is in direct proportion to the quantity of food given and inverse to the prolongation of larval and to the abbreviation of pupal life. (The decrease of weight of caterpillars is larger in cases of longer though more rare food-intervals than in cases of more frequent but shorter ones.) These rules may be applied only to differently starved whole experimental materials, having no application to separate specimens of separate broods, both starved and control.

6. The capacity to grow as well as the capacity to undergo metamorphosis exists in starved specimens far beyond the age at which control caterpillars cease to grow and undergo transformation.

7. During long lasting starvation organisms get accustomed to the abnormal conditions: the rate of growth of the caterpillars starved every second day during their whole life becomes in time considerably greater than that of specimens analogically deprived of food since their last moult but one.

8. Hunger death of caterpillars is probably caused first of all by exhaustion of reserve substances. Natural death of the imago probably is a function of the character of metabolism, as death is delayed by the changed metabolism of intermittent starvation.

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# ON THE HETEROGENEOUS INFLUENCE OF STARVATION OF MALE AND OF FEMALE INSECTS ON THEIR OFFSPRING.

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(WITH 4 TABLES.)

Our knowledge of the influence exerted by insufficient feeding of the parents on their offspring is almost entirely confined to certain observations made on fetuses of the Mammalia. It has been noticed that these fetuses are able to take from tissues of the mother the substances which are more or less lacking in her food. The problem as to whether and to what degree starvation of either of the parents separately has any influence on the total development of their progeny from birth till sexual maturity has so far as I know, not as yet been methodically investigated.

Kellogg and Bell ('04) ascertained that starved silkworms mated with each other produce caterpillars which develop more slowly and produce lighter moths, in spite of the normal feeding conditions of this generation. But, as the authors have exclusively mated the starved specimens with each other they have not discriminated the influence of starvation of either of the parents separately. For this discrimination it was necessary to mate both females subjected to starvation with control males and normal females with starved males. These two experiments were undertaken in the present research.

The experiments were performed on the moth *Lymantria dispar* L. The whole material came from series *D* of my investigations on the influence of starvation of caterpillars on the development of insects, (Kopeć, '21 and '23). As *Lymantria dispar* does not take any food in its imaginal stage, starvation of the parents can take place only during their larval stage. In that series of experiments the caterpillars were fed every second day since their last moult but one. Adult moths which develop from these

[1] Paper from the laboratory of Experimental Morphology. Cf. *Mém. de l'Institut Nat. Polonais d'Économie Rurale à Pulawy*, Vol. 2, 1922.

caterpillars were mated to control moths which were at my disposal. In the filial generation the conditions of rearing were identical in each lot. Food was given daily and in superfluity. As all lots originated from one pair of moths caught in 1917, the material is to a certain degree genetically homogeneous.

The following physiological characters of the filial generation were taken into account: (1) The duration of the larval life, which in the parental generation had undergone considerable prolongation owing to inanition, (2) the duration of the pupal stage which in the parents had undergone a certain abbreviation, (3) the limits of growth of the caterpillars expressed by the weight of fresh pupæ which in the starved parental generation were much smaller than control specimens. Apart from this, the size of the eggs out of which the first filial generation developed, their number and their capacity to develop as well as the mortality of caterpillars and of chrysalids have been examined. The changes of the above-mentioned characters were in the filial generation in part much smaller than in the parents. I therefore did not use for my calculations in these experiments the averages of a character for each lot separately, but in each experiment I joined all specimens of either sex of all lots into class-frequencies as usual and I calculated the average values for all specimens together with the probable error of these averages ($M \pm E_1$). On comparing any character of the control and of the experimental material only such differences are considered as essential, *i.e.*, biometrically well grounded, which are larger than four times their probable error $\left( \frac{\text{Diff.}}{E_{\text{Diff.}}} > 4 \right)$. When the ratio $\frac{\text{Diff.}}{E_{\text{Diff.}}}$ is $< 4$ the differences are considered as biometrically not essential and the character as not changed.

### The Offspring of Females Subjected to Starvation Mated with Control Males.

From Table I. we may infer that the number of eggs laid by these specimens was much smaller than that produced by control females mated with control males. On the other hand, the size of the eggs was biometrically identical in both cases, as is evidenced in Table II. Almost all eggs from starved females were capable of development, although a large number of the develop-

TABLE I.

| | Offspring of Control Females Mated with Control Males. | | | | Offspring of Starved Females Mated with Control Males. | | | | Offspring of Control Females Mated with Starved Males. | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Number of Eggs. | Per Cent. of Eggs from which Caterpillars Hatched. | Per Cent. Caterpillars which Under-went Pupation. | Per Cent. Chrysalids from which Moths Emerged. | Number of Eggs. | Per Cent. of Eggs from which Caterpillars Hatched. | Per Cent. Caterpillars which Under-went Pupation. | Per Cent. Chrysalids from which Moths Emerged. | Number of Eggs. | Per Cent. of Eggs from which Caterpillars Hatched. | Per Cent. Caterpillars which Under-went Pupation. | Per Cent. Chrysalids from which Moths. Emerged. |
| | 197 | 48.7 | 20.8 | 100.0 | 69 | 31.8 | 31.8 | 100.0 | 346 | 90.7 | 10.1 | 68.7 |
| | 346 | 61.2 | 29.7 | 74.6 | 107 | 53.2 | 29.8 | 94.1 | 453 | 94.7 | 0.9 | 75.0 |
| | 295 | 52.5 | 52.2 | 93.8 | 268 | 46.9 | 5.6 | 85.7 | 377 | 83.2 | 2.5 | 75.0 |
| | 348 | 42.5 | 27.0 | 85.0 | 262 | 51.1 | 32.0 | 75.0 | 335 | 86.2 | 12.1 | 65.7 |
| | 160 | 75.0 | 27.5 | 87.8 | 177 | 45.1 | 32.5 | 88.4 | 376 | 78.4 | 4.0 | 75.0 |
| In | 242 | 82.6 | 5.0 | 90.0 | 80 | 25.0 | 35.0 | 71.4 | 319 | 25.0 | 2.5 | 50.0 |
| separate | 277 | 94.6 | 28.6 | 86.6 | 99 | 90.9 | 55.5 | 76.0 | 411 | 90.9 | 15.5 | 70.5 |
| lots | 381 | 50.1 | 27.7 | 92.4 | 129 | 68.9 | 20.2 | 88.8 | | | | |
| | | | | | 222 | 89.6 | 25.1 | 84.0 | | | | |
| | | | | | 49 | 51.0 | 24.0 | 83.3 | | | | |
| | | | | | 97 | 82.4 | 68.0 | 98.0 | | | | |
| | | | | | 102 | 78.4 | 62.5 | 96.0 | | | | |
| | | | | | 57 | 24.5 | 50.0 | 71.4 | | | | |
| Average.. | 280 7 | 63.4 | 27.3 | 88.7 | 132.1 | 56.8 | 36.3 | 85.5 | 373.8 | 78.4 | 6.8 | 68.5 |

ing eggs died in time. (The number of eggs which did not develop at all was not larger than 1 per cent. of all eggs in the control material as well as in that experimented upon.) The percentage of eggs from which caterpillars hatched was approximately identical in this experimental material and in the control specimens. The same may be said as to the percentage of caterpillars which

TABLE II.

A—average; $E_A$—probable error of the average; $\sigma$—standard deviation; $n$—number of specimens; Diff.—difference between the control and the experimental material; $E_{Diff.}$—probable error of this difference.

DATA CONCERNING MAXIMAL DIAMETERS OF EGGS, IN $\mu$.

| Materials. | $A \pm E_A$. | $\sigma$. | $n$. |
|---|---|---|---|
| Eggs of control females mated with control males | 1234.65 ± 2.319 | 53.81 | 245 |
| Eggs of starved females mated with control males | 1237.09 ± 1.783 | 51.61 | 381 |
| Eggs of control females mated with starved males | 1238.00 ± 2.064 | 44.35 | 210 |

DIFFERENCES OF THE ABOVE AVERAGES, IN $\mu$

| Averages Compared. | Diff. $\pm E_{Diff.}$ | $\frac{Diff.}{E_{Diff.}}$ | Nature of Difference. |
|---|---|---|---|
| Difference between the average size of control eggs and of that of the eggs from starved females mated with control males. | −2.44 ± 2.925 | 0.83 | Not essential. |
| Difference between the average size of control eggs and of that of the eggs from control females mated with starved males. | −3.35 ± 3.104 | 1.08 | Not essential. |

underwent pupation and of the chrysalids which developed into adult moths (cf. Table I.). Pupation of caterpillars and hatching of moths having been checked once daily during one and the same hour, the limits of this life-period were determined with exactness up to 24 hours. The data of Tables III. and IV. referring to the duration of the larval and of the pupal life in the progeny of starved females mated with control males as well as the same data of the control material (control ♀♀ × control ♂♂) show that the duration of the larval life is not essentially changed but the duration of the pupal period of the offspring of the females subjected to starvation has undergone essential abbreviation. The limits of larval growth have been determined, as in the

experiments with the parental generation, by the weight of new-formed chrysalids. As pupation was checked once a day it ought to be emphazised that during the first 24 hours the chrysalid did not lose more than 0.15 to 0.28 per cent. of the original weight of the chrysalid immediately after its emergence from the larval skin. If we take into consideration the appertaining strict biometrical data of Tables III. and IV., as well as the ratio $\frac{\text{Diff.}}{E_{\text{Diff.}}}$, we may ascertain that the weight of the chrysalids underwent no

## Table III.

Data Referring to the Duration of Larval and Pupal Life and to the Weight of Chrysalids.

$A$—average; $E_A$—probable error of the average; $\sigma$—standard deviation; $n$—number of specimens.

| | Materials. | Sex of the Specimens. | $A \pm E_A$. | $\sigma$. | $n$. |
|---|---|---|---|---|---|
| Duration of larval life in days. | Caterpillars from control females mated with control males. | ♂♂ | 52.81 ± 0.247 | 4.42 | 146 |
| | | ♀♀ | 63.21 ± 0.309 | 6.93 | 229 |
| | Caterpillars from starved females mated with control males. | ♂♂ | 51.66 ± 0.217 | 4.23 | 173 |
| | | ♀♀ | 63.73 ± 0.320 | 6.14 | 167 |
| | Caterpillars from control females mated with starved males. | ♂♂ | 52.22 ± 0.325 | 4.57 | 99 |
| | | ♀♀ | 63.39 ± 0.762 | 9.52 | 71 |
| Duration of pupal life in days. | Chrysalids from control females mated with control males. | ♂♂ | 22.83 ± 0.0568 | 0.93 | 122 |
| | | ♀♀ | 19.15 ± 0.0525 | 1.12 | 207 |
| | Chrysalids from starved females mated with control males. | ♂♂ | 22.21 ± 0.0521 | 0.93 | 145 |
| | | ♀♀ | 18.51 ± 0.0420 | 0.70 | 149 |
| | Chrysalids from control females mated with starved males. | ♂♂ | 21.91 ± 0.0771 | 0.84 | 54 |
| | | ♀♀ | 18.45 ± 0.1275 | 1.44 | 58 |
| Weight of chrysalids in mg. | Chrysalids from control females mated with control males. | ♂♂ | 283.56 ± 4.63 | 82.92 | 146 |
| | | ♀♀ | 758.51 ± 13.04 | 292.64 | 229 |
| | Chrysalids from starved females mated with control males. | ♂♂ | 303.76 ± 4.17 | 81.38 | 173 |
| | | ♀♀ | 794.61 ± 18.02 | 345.25 | 167 |
| | Chrysalids from control females mated with starved males. | ♂♂ | 216.67 ± 4.14 | 58.21 | 99 |
| | | ♀♀ | 609.86 ± 19.34 | 241.65 | 71 |

## TABLE IV.

DIFFERENCES OF AVERAGE DURATION OF LARVAL AND OF PUPAL LIFE AS WELL AS OF WEIGHT OF CHRYSALIDS BETWEEN THE CONTROL AND THE EXPERIMENTAL MATERIAL.

Diff.—difference between the control and the experimental material. $E_{Diff.}$—probable error of this difference.

| | Averages Compared. | Sex of the Specimens. | Diff. ± $E_{Diff.}$ | $\frac{Diff.}{E_{Diff.}}$ | Nature of Difference. |
|---|---|---|---|---|---|
| Data concerning larval life, in days. | Difference between the average duration of larval life of control specimens and of that of specimens deriving from starved females mated with control males. | ♂ ♂ | 1.15 ± 0.329 | 3.49 | Not essential. |
| | | ♀ ♀ | − 0.52 ± 0.445 | 1.17 | " |
| | Difference between the average duration of larval life of control specimens and of that of specimens deriving from control females mated with starved males. | ♂ ♂ | 0.59 ± 0.408 | 1.44 | Not essential. |
| | | ♀ ♀ | − 0.18 ± 0.822 | 0.22 | " |
| Data concerning pupal life, in days. | Difference between the average duration of pupal life of control specimens and of that of specimens deriving from starved females mated with control males. | ♂ ♂ | 0.62 ± 0.0771 | 8.04 | Essential. |
| | | ♀ ♀ | 0.64 ± 0.0672 | 9.52 | " |
| | Difference between the average duration of pupal life of control specimens and of that of specimens deriving from control females mated with starved males. | ♂ ♂ | 0.92 ± 0.0958 | 9.60 | Essential. |
| | | ♀ ♀ | 0.70 ± 0.1378 | 5.08 | " |
| Data concerning weight of chrysalids, in mg. | Difference between the average weight of control pupæ and of that of pupæ deriving from starved females mated with control males. | ♂ ♂ | −20.20 ± 6.23 | 3.24 | Not essential. |
| | | ♀ ♀ | −36.10 ± 22.24 | 1.62 | " |
| | Difference between the average weight of control pupæ and of that of pupæ deriving from control females mated with starved males. | ♂ ♂ | 66.89 ± 6.21 | 10.77 | Essential. |
| | | ♀ ♀ | 148.65 ± 23.32 | 6.37 | " |

essential change in this experimental material. (The pupæ are here even heavier, but the differences are smaller than their fourfold error.)

### The Offspring of Control Females Mated with Males Subjected to Starvation.

As the control females mated with starved males were by chance mostly larger than the control females mated with control males, they laid somewhat larger quantities of eggs (Table I.). The egg size, however, was identical in this material and in that of the control, as may clearly be inferred from Table II. Also in this experiment the percentage of eggs which did not develop at all was not larger than 1 per cent. of all eggs.

An exact inquiry into the data of Table I. shows that the capacity of the spermatozoa from starved males to fertilize and to timulate eggs to total development is, if not larger, at least nots smaller than in spermatozoa of control specimens. On the other hand, mortality of caterpillars as well as of chrysalids in this experiment was undoubtedly larger than in control materials. From data of Tables III. and IV. we see that the larval life in the offspring of control females mated with males subjected to inanition does not undergo any essential change. On the other hand, the duration of the pupal stage is essentially shortened in this experiment. On comparing the data of the same tables it is also found that in contrast to the offspring of starved females mated with control males, the offspring of males subjected to starvation and of control females is lighter than the progeny of control material.

### General Conclusions and Summary.

The above experiments are the first to discriminate strictly the influence of starvation exerted on the offspring by either of the parents separately. The starved females the progeny of which was used for this research were much smaller than the control specimens, their limit of growth being only 48.4 per cent. of the normal limit of growth (Kopeć, '21 and '23, Table III.). Nevertheless, the body-weight of the offspring of such starved females was at least normal. This leads to the supposition that in females hunger metabolism does not bring about any quantitatively

unfavorable changes in the reserve substances out of which eggs are developed in the chrysalids. On the other hand, we have to do here with a special regulation of quantitative relations, consisting of two factors: the number of eggs laid by the females undergoes in general considerable reduction, but the egg size does not exhibit any changes, which points to unchanged quantity of food substances for the developing embryo in each "starved" egg.

The normal size of eggs layed by starved females together with the decreased number of the eggs speak in favor of the supposition that in case of insufficient feeding of an animal the food substances may be exclusively used up by a certain number of eggs which develop better than the remaining, owing to their arrangement in the ovary or to other conditions. This is caused by the competition between separate elements, which undoubtedly takes place in the starved ovary analogous to the struggle between individuals of a brood, which develops in unfavorable conditions. Owing to this rivalry, only part of the eggs undergo development, directly or indirectly at the expense of neighboring elements, as the number of eggs contained in "starved" chrysalids is considerably larger than the number of fully developed eggs in the mature females. Notwithstanding the capacity of certain "starved" eggs to develop at the expense of other eggs, this average size does not exceed essentially the normal average limit, which points to the dependence of egg size on certain internal factors by which the limits of the growth of eggs are determined. These factors are undoubtedly synonymous with "genes" which determine in genetical sense the size of eggs independently of the size of the females by which the eggs are produced. In spite of this evolutive independence during the development of a fetus or of an egg, there probably exists a certain limit of degree and of duration of starvation which may be applied to a female, beyond which limit the progeny does not attain its normal size. The assumption that this limit may easily be transgressed is supported by the fact that certain small unfavorable changes in feeding birds cause a considerable diminution of their eggs and even check the further processes of development of the sexual elements. This limit was undoubtedly transgressed in the experiments made by Woltereck ('08–'11,) in which the

offspring parthenogenetically produced by starved *Daphniæ* was smaller than control specimens.

In contrast to the offspring of starved females the progeny of similarly treated males were essentially lighter than those produced by control males. The unfavorable influence of starvation of males was evidenced by increased mortality of caterpillars as well as of chrysalids, which could not be observed in the offspring of starved females. As egg size was also in this experiment normal, the cause of these changes ought to be ascribed to the specific influence exerted by starvation of males on their spermatozoa.

A great influence on the offspring of the Mammalia exerted by certain substances applied to their fathers was demonstrated, *e.g.*, by experiments on the influence of alcohol made by Stockard and his collaborators ('12–'18) and others, by the experiments on lead performed by Cole and Bachhuber ('14) and by those of Guyer and Smith ('18 and '20) on a special lens dissolving serum. These researches have shown a considerable susceptibility of the spermatozoa to certain extrinsic specific substances. My experiments prove that normal development of the spermatozoa may also be influenced by certain natural changes of metabolism caused by inanition. Histological research has shown that the males which develop from starved caterpillars contain the same quantities of reserve substances relatively to their diminished size as control specimens; as the testicles of the moth which develop in the caterpillar at the expense of these substances are too small organs to play an important rôle in the nutritive balance of the chrysalis undergoing metamorphosis, it ought to be supposed that the influence exerted on the spermatozoa by hunger metabolism consists in qualitative and not in quantitative changes of the chemical constitution of the spermatozoa. In my experiments I did not succeed in ascertaining any marked histological changes in the developing "starved" testicle, neither as to the evolutive rate, nor as to the structure of the sexual elements. Physiological dimorphism of metabolism in the two sexes is certainly the cause why starvation of females does not elicit analogous unfavorable quantitative changes in the egg.

From the above results we may infer that the decreased weight of the offspring of two starved parents determined in silkworms

by Kellogg and Bell ('04) was exclusively due to the influence of the spermatozoa from the starved males, unless the limits beyond which we ought to expect a noxious influence of the starvation of females on their progeny had been transgressed, owing to different degree of starvation. It ought to be mentioned that in certain species the development of the sexual elements occurs in natural conditions during periods of total physiological starvation. Miescher ('97) ascertained that in the salmon which does not take any food during the development of its sexual glands its very large ovaries and testicles grow at the expense of muscles. This fact, however, is in no discrepancy with my results as it may be that certain special feeding experiments with salmon—if they are practically possible—would, as in moths, elicit in this fish changes as to the size of the offspring which in natural conditions develops here from "starved" spermatozoa. The generalization of my results obtained in insects to other animals, especially to vertebrates, would be premature. I nevertheless believe that my present contribution will have a certain general interest in regard to the great biological importance of the discussed problem.

The duration of the larval period does not undergo any essential change in the offspring of starved females nor in that of the starved males either. The pupal stage, on the contrary, is essentially changed in both cases. My conception of the physiology of insect metamorphosis is supported by such behavior of the progeny. According to my opinion the larval brain elicits by its secretion or secretions the histolysis of larval tissues, which puts an end to larval life (Kopeć, '17 and '22). It also checks in the larva the development of the embryonal discs from which the organs of the imago are finally formed in the chrysalids ('21 and '23). The prolongation of the larval life and the simultaneous abbreviation of the pupal stage caused by starvation of caterpillars has been interpreted as due to delayed or decreased secretoric function of the brain. If the prolongation of larval life elicited by starvation of the mother or of the father is due to delayed or decreased production of the substance by which histolysis of tissues is brought about, no wonder that in the filial generation there is no change in respect to the duration of larval life: in these experiments the larval organism of the filial generation remains unchanged as to its preparation for histolysis. The

duration of the pupal stage in the filial generation must be on the contrary explained in a different manner. It has been shown that the abbreviation of the pupal period and consequently the acceleration of development of the embryonal discs was brought about even by temporary starvation of very young caterpillars, viz., lasting 20 days after hatching from eggs, *i.e.*, at a time when there are in the larval organism not yet any traces of histolytical processes characteristic of pupation (Kopeć, '21 and '23). It may therefore easily be assumed that not only the embryonal discs of the animal subjected to inanition, but also the evolutive factors contained in their sexual cells, which cause the evolution of the embryonal discs in the filial generation, are stimulated to development by starvation and by the decreased checking function of the brain connected therewith. In the caterpillars of this filial generation the brain finds therefore the embryonal discs which are more physiologically advanced and this fact eventually leads to a certain abbreviation of the pupal stage of the progeny of starved females or of starved males.

On surveying the results of the foregoing inquiry the following summary may be given:

1. The females of the moth *Lymantria dispar* L. derived from starved caterpillars mated with control males laid smaller quantities of eggs than control specimens, but the dimensions of the eggs and the weight of the pupæ developing from them did not undergo any essential changes. The capacity of the egg to develop and the mortality of caterpillars as well as of chrysalids are not changed by the starvation applied in these experiments as compared with control material.

2. The morphological structure of the spermatozoa of males deriving from similarly starved caterpillars is unchanged and they have the normal capacity for stimulating eggs to development, but the mortality of the resulting caterpillars and chrysalids is distinctly larger than that of the control material. The weight of the pupæ in the progeny of starved males undergoes essential decrease.

3. Distinctly injurious effects were consequently brought about in the offspring by inanition of the males, in contrast to inanition of the females. The causes of the different behavior of either of the starved parents ought to be referred to metabolism probably

different in the two sexes. Changes elicited in the structure of the spermatozoa by starvation of males are most probably qualitative.

4. The prolongation of larval life which had been noticed in the starved male as well as female caterpillars could not be ascertained in their offspring. The pupal stage, on the contrary, which had been much shortened in starved specimens underwent in the offspring of starved females as well as in the offspring of starved males an analogous change which leads to acceleration of metamorphosis. Such behavior of the progeny of starved specimens supports the author's former opinion that insect metamorphosis is checked by the secretoric function of the larval brain.

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# A NEW FIELD METHOD OF INVESTIGATING THE HYDROTROPISMS OF FRESH-WATER INVERTEBRATES.

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## Introduction.

During the past quarter of a century the tropism idea has stimulated intensive investigation of the factors that serve as directive forces in the behavior of animals. Gravitation, light, heat, contact, wind and water currents, as factors of orientation, have been much investigated; but, the directive influence of bodies of water as such has received scant attention. In her learned textbook,[3] Professor Washburn does not discuss the subject. In his book on animal intelligence [2] Professor Holmes devotes a whole chapter to tropisms. He discusses; chemotaxis, geotaxis, thigmotaxis, rheotaxis, phototaxis, thermotaxis and electrotaxis; but, not a word about hydrotaxis. Professor Bouvier, in a recent work,[4] says more on this topic than I have seen in any other book on animal behavior. He writes:

"No less than heat, water is necessary to living beings, for it constitutes the greater portion of their protoplasm and plays a part in almost all their internal changes. Also, all organisms are sensitive to variations of humidity in the space surrounding them, and with a great number this sensitiveness takes the form of a directive orientation which is called hydrotropism.

"We know the famous experiments made by Stahl, in 1884, on the hydrotropism of the fungi of the genus *Æthalium* (tanning fungus). The plasmodial mass of these plants, during the vegetation period, enters the tan, making for the humidity necessary

1. *Note by the editor.* This paper was received for publication January 28, 1923. The author died February 14, 1923. Dr. Turner was a student of Professor C. O. Whitman, and received the degree of Doctor of Philosophy from the University of Chicago in 1907. His thesis was on "The Homing of Ants: An Experimental Study of Ant Behavior." He continued his patient, thorough studies of animal behavior to the time of his death, as the present contribution will testify. Several studies by Dr. Turner have appeared in the pages of the Biological Bulletin.

to it, and remounts to the surface in a dry *milieu* when it is going to form its spores which serve its multiplication. Its hydrotropism is positive. This is the case with the beetles of the genera *Haliplus* and *Hydroporus*. Wheeler (1899) had taken from a pool a tuft of aquatic plants where these insects swarm. He says: 'As soon as the beetles could come out and disengage themselves from the plant they turned, with a common accord, toward the sea and to it directed their steps. As this was a distance of about twenty feet, the little creatures could not see the water, and I was led to believe that they had some means of perceiving a source of moisture and acted accordingly.

"Aquatic bugs act the same way when they are taken from the place in which they live, and we know that the land crabs go a long distance to water when they are ready to place their progeny. The proper degree of humidity differs, moreover, with different species. Wheeler reports that *Bembidium*, *Elaphrus*, *Omophron*, and other small Coleoptera which bury themselves in the sandy beaches, leave their burrows and come out into the open air when one throws a little water on their strand. This is negative hydrotropism. It is well known to collecting entomologists, who use it in making captures."

Weiss [4] reports two cases of what he calls positive hydrotropism. When specimens of the wingless *Gerris marginatus* were removed one to nine yards from the pond they immediately returned to it. When removed ten yards from the water they had some trouble in getting started in the right direction; but finally reached the pond. Thirty yards from the water they seemed to be lost. The case of *Dineutes assimilis*, a winged beetle, is even more interesting. When removed nine or ten feet from the pond it tried to walk to the pond, then arose and flew directly to it. When removed to a distance of seventy-five feet, it walked about in all directions, then arose, on its wings, to a height of twenty feet and flew directly to the water. When removed half a mile from the pond, it soared in a widening sub-spiral to a height of seventy-five feet and then flew off in the direction of the water. He does not know whether it reached the water or not.

It is not claimed that the above resumé contains all that has been written on this topic. It is quite likely that some articles have been overlooked. However, the fact that a diligent search

has supplied only this suggests that very little attention has been paid to the topic. This may be because no simple means of investigating it has been published. The purpose of this paper is to supply that lack and to apply the method to the study of a few forms selected from different strata of the water. As types of forms that creep along the bottom of the water or along vegetation but do not leave the water, I selected a snail [*Planorbis* (*Helisoma*) *antrosum* Con.] and a dragon-fly nymph. As examples of forms that spend most of their time on the bottom, but which, at times, leave the water, I have slected the crayfish [*Cambarus* (*Faxonius*) *propinquus* Girard] and a scavenger beetle (*Tropisternus* sp. ?). As types of forms that live on the surface of the water, I have selected the giant water strider (*Gerris remigis* Say), which, except during the hibernating season, is practically a permanent inhabitant of the film, and the whirligig beetle (*Gyrinus* sp.?) which occasionally dives and which migrates from pond to pond.

## Technique.

To be able to study the movements of animals with a certainty that the movements were not influenced by either gravitation, the sun's rays, chemical stimuli or contact stimuli, I devised what may be appropriately called a checker-board plate. This consists of a board twenty inches wide and twenty-four inches long which is covered with white oilcloth that has been subdivided, by printed lines, into one inch squares (Fig. 1). The four edges of the board were bisected by straight lines which crossed, at right-angles at the center of the board. Diagonal lines, bisecting these central right angles extended outward to the edges of the board. The tips of the four lines mentioned were named; 0 degrees, 45 degrees, 90 degrees, 135 degrees, 180 degrees, 225 degrees, 270 degrees, 315 degrees. By means of a level and leveling devices this board was made perfectly horizontal. Being horizontal, gravitation could not influence the movements. Being an absolutely smooth surface, the movements could not be influenced by contact stimuli. Being covered with oil-cloth, it was possible, by washing, to keep it free from chemical stimuli. It was protected from the sun by means of a screen of some sort; this made it impossible for the sun's rays to influence

the movements. The checker-board pattern made it easy to watch the movements of the animals.

Using a scale of one-eighth of an inch equals an inch sheets of cross section paper were cut to resemble the board, and the tips of radii labeled in the same manner as on the board. These were carried to the field in a loose-leaf cover.

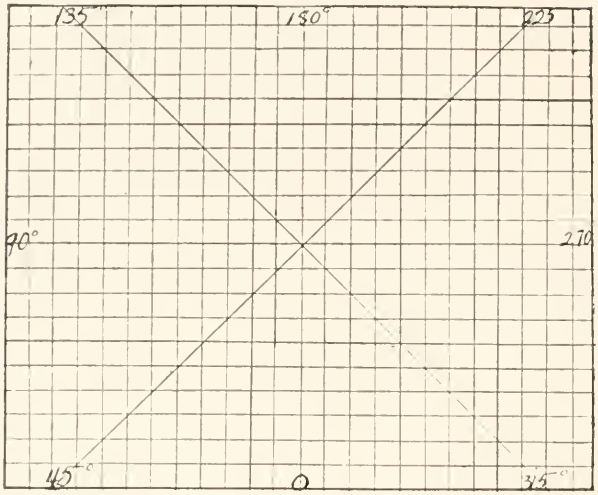


FIG. 1. Checkerboard Plate.

At the beginning of a series of experiments the board is placed on the ground and adjusted until it is perfectly horizontal. If necessary, it is screened from the sun. One of the sheets is then numbered to correspond with the specimen to be investigated. On the reverse side is recorded the position of the sun, the position of the water and the direction in which the animal's head is to be placed. In describing these positions the labeled tips of the radii are used as reference points. The animal is then placed in the center of the board with its head in the desired direction and its movements traced on the cross-section paper. When the animal reaches the edge of the board, it is returned to its original position. This is repeated until it has reached the edge of the board five times. It is then replaced in the water a short time and then returned to the center of the board with its head facing a new position. It is allowed to make five trips in that case; these are recorded on a new sheet. This

is repeated over and over again, using one sheet of paper for each five experiments, until the creature has worked with its head facing all eight of the radii tips. This gives forty experiments and is called a series. Work is continued until one has secured records of as many series as is thought necessary. In the work recorded in this paper an attempt was made to secure between twelve and thirteen series with each species investigated.

In practice it is not always possible to secure a complete series from an individual. Sometimes the individual would escape before the series was completed, at others fatigue would put an end to the experiments at an early stage. In some cases it was necessary to return the specimen to the water at the close of each experiment, instead of at the close of each five. Two investigators can do more effective work than one; one giving his entire time to looking after the animal and the other recording what happens. This is especially important when dealing with forms that fly and with crawling forms that move rapidly. After a little experience one recognizes the movements preliminary to flight. Usually a small glass cover placed over the specimen for a moment will check the attempt to fly.

In the work performed in connection with this article, on returning from the field the results of the days work were recorded on tables with the following captions;

TABLE I.

| Name of Species. | Number of Specimen. | Position of Sun. | Position of Water. | Head Pointed towards. | Number of Trials | Nature of the Water. | Time of Day. | Final Movement. | | | | | | Sex. | Age. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Toward Water. | Not toward Water. | Toward Sun. | Not toward Sun. | Way Head Pointed. | Not Way Head Pointed. | | |
| | | | | | | | | | | | | | | | |

Later these tables were condensed into those published in this article.

In the experiments recorded in this article the side of the

board marked O, always faced the water. If there had been an unlimited number of experiments, twenty-five per cent of movements toward the water would indicate that the movements were random and not influenced by the position of the water. Since the number of experiments was limited to about 500, unless the movements toward water were much more than twenty five per cent. the movements were considered random. In recording movements toward the position of the sun and the initial position of the insect's head, eight possible directions were considered. In each of these cases, if there had been an infinite series of experiments, 12.5 per cent. of movements in a certain direction would indicate random movements. Hence, since there were only about 500 experiments, unless the per cent of movements in a certain direction was much more than 12.5 the movements were considered random.

With the animals investigated in this connection, in addition to the experiments with the checker-board plate, the creatures were set free, at definite distances from the water, and their movements watched. If the experiments demonstrated a tendency to move toward the water, then the specimens were blinded and the experiments repeated.

### Dragon-fly Nymphs (Plate I).

In experimenting with the checker-board plate fourteen individuals were used. In some cases the board was ten feet from the water; in others it was fifteen. Five hundred and fifty-six experiments were performed. The results are recorded in Table II.

In an infinite series of experiments, if the factors considered did not influence the movements in any manner, 25 per cent. of the movements should be toward water, 12.5 per cent. toward the position of the sun and 12.5 per cent. to the way the head originally pointed. The actual results were 25 per cent toward water, 20.5 per cent. toward the position of the sun and 23.3 per cent. toward the way the head originally pointed. Only three individuals made more than thirty per cent. of movements to water. Evidently, dragon-fly nymphs, when moving about on the checker-board plate are not influence by the nearness of bodies of water.

TABLE II.

DRAGON-FLY NYMPHS.

| Specimen Number. | Number of Trials. | Kind of Water. | Initial Positions. | Per Cent. of Final Movements. | | | | | | Time of Day. |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Toward Water. | Not toward Water. | Toward Sun. | Not toward Sun. | Way Head Pointed. | Not Way Head Pointed. | |
| 1 | 10 | Pond | 2 | 10 | 90 | 10 | 90 | 30 | 70 | A.M. |
| 2 | 3 | Pond | 1 | 0 | 100 | 100 | 0 | 0 | 100 | A.M. |
| 3 | 5 | Lake | 1 | 100 | 0 | 0 | 100 | 0 | 100 | A.M. |
| 4 | 18 | Pond | 4 | 29 | 71 | 11 | 89 | 33 | 67 | P.M. |
| 5 | 43 | Lake | 8 | 30 | 70 | 21 | 79 | 30 | 70 | A.M. |
| 6 | 5 | Pond | 1 | 0 | 100 | 0 | 100 | 40 | 60 | P.M. |
| 7 | 80 | Pond | 8 | 49 | 51 | 37 | 63 | 21 | 79 | P.M. |
| 8 | 40 | Pond | 8 | 7 | 93 | 0 | 100 | 25 | 75 | P.M. |
| 9 | 80 | Pond | 8 | 17 | 83 | 15 | 85 | 28 | 72 | Both |
| 10 | 40 | Pond | 8 | 22 | 78 | 5 | 95 | 15 | 85 | A.M. |
| 11 | 167 | Pond | 8 | 57 | 43 | 0 | 100 | 11 | 89 | Both |
| 12 | 10 | Pond | 2 | 10 | 90 | 20 | 80 | 20 | 80 | A.M. |
| 13 | 25 | Pond | 5 | 12 | 88 | 48 | 52 | 24 | 76 | P.M. |
| 14 | 30 | Pond | 4 | 10 | 90 | 20 | 80 | 50 | 50 | A.M. |
| Total | 556 | Av.- | 4.8 | 25 | 75 | 20.5 | 79.5 | 23.3 | 76.7 | |

NORMAL CRAYFISH [*Cambarus* (*Faxonius*) *propinquus* GIRARD] (PLATE II).

In experimenting with the checker-board plate fourteen individuals were used, varying in length from one and a half inches to three inches. In most cases the board was placed within five or ten feet of the water, in some cases it was forty feet from the water and, in a very few cases, it was fifty feet away. In most cases the experiments were conducted near the river or brook from which the crayfish were obtained. In others the crayfish were carried to the borders of some lake or pond. The results are recorded in Table III.

In an infinite series of experiments twenty-five per cent. of movements toward the water is what should be expected if the water has not directive influence on the individuals. The experiments yielded 67.5 of movements toward the water. Three individuals made 100 per cent. of movements toward the water, four between 90 and 100 per cent. In only four cases did the per cent. fall under 50 and the lowest of those was 36. Evidently

this crayfish, when moving on the checkerboard plate, is largely directed by the nearest body of water. It acts as though it were positively hydrotropic.

TABLE III.

THE CRAYFISH [*Cambarus* (*Faxonius*) *propinquus* Girard].

| Specimen Number. | Number of Trials. | Size. | Kind of Water. | Sex. | Initial Positions. | Per Cent. of Final Movements. | | | | | | Time of Day. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Toward Water. | Not Toward Water. | Toward Sun. | Not Toward Sun. | Way Head Pointed. | Not Way Head Pointed. | |
| 1 | 13 | 1¼ in. | Brook | fem. | 6 | 38 | 62 | 15 | 85 | 23 | 77 | A.M. |
| 2 | 7 | 1 in. | Brook | fem. | 2 | 100 | 0 | 0 | 100 | 0 | 100 | A.M. |
| 3 | 3 | 1 in. | Brook | fem. | 1 | 100 | 0 | 0 | 100 | 0 | 100 | A.M. |
| 4 | 80 | 2 in. | Pond | male | 8 | 41 | 59 | 1 | 99 | 11 | 89 | Both |
| 5 | 40 | 1 in. | Pond | fem. | 8 | 97 | 3 | 0 | 100 | 0 | 100 | P.M. |
| 6 | 40 | 1 in. | Lake | fem. | 8 | 75 | 25 | 30 | 70 | 22 | 78 | P.M. |
| 7 | 40 | 1 in. | Lake | fem. | 8 | 100 | 0 | 2 | 98 | 15 | 85 | P.M. |
| 8 | 40 | 1¼ in. | Lake | fem. | 8 | 65 | 35 | 13 | 87 | 8 | 92 | P.M. |
| 9 | 40 | 3 in. | Pond | male | 8 | 52 | 48 | 0 | 100 | 28 | 72 | P.M. |
| 10 | 80 | 1½ in. | Pond | fem. | 8 | 91 | 9 | 3 | 97 | 23 | 77 | A.M. |
| 11 | 7 | 1½ in. | Pond | fem. | 2 | 57 | 43 | 0 | 100 | 14 | 86 | A.M. |
| 12 | 80 | 2½ in. | River | fem. | 8 | 56 | 44 | 7 | 93 | 35 | 65 | Both |
| 13 | 45 | 1 in. | River | male | 8 | 36 | 64 | 11 | 89 | 29 | 71 | A.M. |
| 14 | 40 | 1 in. | River | male | 8 | 38 | 62 | 15 | 85 | 55 | 45 | A.M. |
| Total | 535 | Average..... | | | 6.5 | 67.5 | 32.5 | 6.9 | 93.1 | 18.8 | 81.2 | |

In an infinite series of experiments, 12.5 per cent. of movements toward the position of the sun would indicate that brightness of the surrounding region (the direct rays of the sun did not have access to the specimens) did not have a directive effect. The experiments yielded only 6.7 per cent. of movements toward the position of the sun. Four individuals made zero per cent. of movements toward the position of the sun, three less than four per cent., in only four cases did the per cent. become more than twelve. Evidently, on the checkerboard plate, the crayfish shows a tendency to move away from the brightest part of the field.

In an infinite series of experiments 12.5 per cent. of movements in the direction the head originally pointed would indicate that the position of the head had not influenced the movements.

These experiments yielded 18.8 per cent. of movements in the direction the head originally pointed. This is so near to what theory demands that it is evident that the original position of the head has not appreciably influenced the final movements of the individual.

In the matters considered no marked difference was observed between males and females.

At the close of each series of experiments mentioned above, the crayfish was placed on the ground, with its head away from the water. The distances from the water ranged from ten to fifty feet. In each case the crayfish finally turned and went to the water, even going around obstacles.

On another occasion twelve crayfish were placed, one at a time, on the ground, within ten feet of the body of water from which they had been obtained. They were always faced away from the water. Eleven of these reached the water in a very few minutes; the twelfth roamed about for fully half an hour before it found the water. Considered alone these experiments would not permit us to draw any conclusions. The land sloped toward the water. The sun was shining. Hence gravity and the sun rays may have influenced the movements. However, the results are in harmony with the conclusions drawn from the experiments on the checkerboard plate; and, when considered in conjunction with some experiments to be described in the next section, they are illuminating.

### Blinded Crayfish (*Cambarus* sp.? and *Cambarus* (*Fraxonius*) *propinquus* Girard) (Plate III).

The above experiments with the checkerboard plate prove conclusively that the nearest body of water, in some manner, forces the crayfish to move towards it. One naturally wonders if the eyes function in this behavior. To test the matter three types of experiments were conducted with blinded crayfish.

In the first series thirteen blinded crayfish were tested on the checkerboard plate. The eyes of five were blinded with an opaque varnish, the eyes of the other eight were amputated. The board was placed twenty feet from the water. The results are recorded in Table IV.

In an infinite series of experiments, if the factors considered did

not influence the movements, 25 per cent. of the movements should be toward water, 12.5 per cent. toward the sun and 12.5 per cent. toward the way the head was originally pointed. The experiments yielded the following results; 25.7 per cent. toward water, 15.3 per cent. toward the sun and 17.1 per cent. toward the way the head originally pointed. Evidently the movements of blind crayfish, on the checkerboard plate, are not influenced by the nearby bodies of water.

TABLE IV.

THE CRAYFISH (*Cambarus*, sp.?) BLIND (PLATE III).

| Specimen Number. | Number of Trials. | Size. | Kind of Water. | Sex. | Initial Positions. | Per Cent. of Final Movements | | | | | | Time of Day. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Toward Water. | Not Toward Water. | Toward Sun. | Not Toward Sun. | Way Head Pointed. | Not Way Head Pointed. | |
| 1 | 40 | 4 in. | Pond | fem. | 8 | 12 | 88 | 0 | 100 | 10 | 90 | P.M. |
| 2 | 20 | 4 in. | Pond | male | 4 | 40 | 60 | 25 | 75 | 5 | 95 | P.M. |
| 3 | 40 | 4 in. | Pond | male | 8 | 17 | 83 | 7 | 93 | 22 | 78 | P.M. |
| 4 | 40 | 4 in. | Pond | fem. | 8 | 42 | 58 | 20 | 80 | 35 | 65 | P.M. |
| 5 | 38 | 4 in. | Pond | male | 8 | 45 | 55 | 11 | 89 | 21 | 79 | P.M. |
| 6 | 40 | 4 in. | Pond | fem. | 8 | 25 | 75 | 15 | 85 | 15 | 85 | P.M. |
| 7 | 40 | 4 in. | Pond | male | 8 | 32 | 68 | 22 | 78 | 17 | 83 | P.M. |
| 8 | 40 | 4 in. | Pond | male | 8 | 20 | 80 | 18 | 82 | 18 | 82 | P.M. |
| 9 | 80 | 4 in. | Pond | male | 8 | 23 | 77 | 29 | 71 | 40 | 60 | A.M. |
| 10 | 40 | 4 in. | Pond | fem. | 8 | 42 | 58 | 13 | 87 | 15 | 85 | A.M. |
| 11 | 65 | 4 in. | Pond | fem. | 8 | 26 | 74 | 9 | 91 | 15 | 85 | A.M. |
| 12 | 10 | 4 in. | Pond | male | 2 | 10 | 90 | 30 | 70 | 10 | 90 | A.M. |
| 13 | 5 | 4 in. | Pond | fem. | 1 | 0 | 100 | 0 | 100 | 0 | 100 | A.M. |
| Total | 498 | Average..... | | | 6.9 | 25.7 | 74.3 | 15.3 | 84.7 | 17.1 | 82.9 | |

These thirteen blinded crayfish were placed on the ground, within fifteen feet of a pond. Their heads were faced away from the water. The land sloped gently towards the water and the sun was shining brightly. At the end of an hour none had reached the water. Several had wandered off into the shrubbery and become lost, three had moved to within two feet of the water and then turned and walked off in another direction, some were wandering about at a greater distance from the water than their original position and three were in practically their original positions.

In another series of experiments the crayfish [*Cambarus* (*Fraxonius*) *propinquus*] were used in pairs. The eyes of one

of each pair were painted with a thick coat of lampblack mixed in liquid glue. The eyes of the other were normal. Twelve pairs were used. They were placed fifteen feet from the water. In ten cases the one with good eyes reached the water in a short time and the one with blinded eyes failed to do so. In one case neither reached the water. In another the one with blinded eyes reached the water and the one with good eyes wandered away from the water. The experiment with this last couple was repeated and yielded similar results.[1] It is probable that the eyes of this blinded crayfish were not entirely blind; because, on the next day, after the eyes had been repainted, this individual behaved like the other blinded ones. Its companion of the first day died over night; hence, it was not possible to re-test it.

These experiments with blinded crayfish, when considered in connection with the experiments with normal crayfish, demonstrate that the stimulus by means of which a body of water controls the movements of the crayfish reach the animal through the eyes.

GIANT WATER BUG [*Belostoma* (*Zaitha*) *flumínea*] (PLATE IV).

The exceptionally dry summer caused many ponds to go dry and it was impossible to secure enough specimens to conduct an extensive number of experiments. Five individuals were used and 109 experiments performed. The checkerboard plate was placed ten feet from the water. The results of these experiments are recorded in Table V.

TABLE V.

GIANT WATER BUGS [*Belostoma* (*Zaitha*) *flumínea*]

| Specimen Number. | Number of Trials. | Age. | Kind of Water. | Initial Position. | Per Cent of Final movements. | | | | | | Time of Day. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Toward Water. | Not Toward Water. | Toward Sun. | Not Toward Sun. | Way Head Pointed. | Not Way Head Pointed. | |
| 1 | 8 | Adult | Pond | S | 70 | 30 | 0 | 100 | 23 | 77 | P.M. |
| 2 | 10 | Juv. | Pond | 2 | 10 | 90 | 0 | 100 | 10 | 90 | P.M. |
| 3 | 40 | Adult | Pond | S | 50 | 50 | 5 | 95 | 30 | 70 | P.M. |
| 4 | 11 | Juv. | Pond | 3 | 73 | 27 | 0 | 100 | 36 | 64 | P.M. |
| 5 | 40 | Juv. | River | S | 32 | 68 | 2 | 98 | 0 | 100 | A.M. |
| Total | 109 | Average..... | | 5.8 | 47 | 53 | 1.4 | 98.6 | 19.8 | 70.2 | |

[1] In this series of experiments two pairs were used at a time; my wife watching one pair while I watched the other.

It is probably unwise to draw conclusions from only a hundred experiments; but, these experiments suggest that the water does have a directive influence upon this giant water bug.

### A Scavenger Beetle (*Tropisternus*) (Plate V).

In experimenting with the checkerboard plate nineteen individuals of this species were induced to perform 571 experiments. In most cases the board was ten to twenty feet from the water. In one case it was fifty feet (number 18); in another case it was 100 feet (number 19). The results of the experiments are recorded in Table VI.

Table VI.

A Scavenger Beetle (*Tropisternus*)—Adults.

| Specimen Number. | Number of Trials. | Kind of Water. | Initial Positions. | Per Cent. of Final Movements. | | | | | | Time of Day. |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Toward Water. | Not Toward Water. | Toward Sun. | Not Toward Sun. | Way Head Pointed. | Not Way Head Pointed. | |
| 1 | 7 | Lake | 5 | 87 | 13 | 0 | 100 | 13 | 87 | A.M. |
| 2 | 2 | Pond | 1 | 50 | 50 | 0 | 100 | 0 | 100 | A.M. |
| 3 | 9 | Pond | 1 | 55 | 45 | 11 | 89 | 11 | 89 | A.M. |
| 4 | 18 | Pond | 3 | 7 | 93 | 0 | 100 | 50 | 50 | P.M. |
| 5 | 40 | Pond | 8 | 3 | 97 | 3 | 97 | 13 | 87 | P.M. |
| 6 | 40 | Pond | 8 | 53 | 47 | 53 | 47 | 20 | 80 | P.M. |
| 7 | 8 | Pond | 1 | 25 | 75 | 25 | 75 | 88 | 12 | P.M. |
| 8 | 40 | Pond | 8 | 0 | 100 | 0 | 100 | 23 | 77 | P.M. |
| 9 | 40 | Pond | 8 | 0 | 100 | 65 | 35 | 25 | 75 | P.M. |
| 10 | 40 | Pond | 8 | 35 | 65 | 0 | 100 | 8 | 92 | P.M. |
| 11 | 40 | Pond | 8 | 55 | 45 | 15 | 85 | 28 | 72 | P.M. |
| 12 | 9 | Pond | 2 | 11 | 89 | 0 | 100 | 44 | 56 | A.M. |
| 13 | 40 | Pond | 8 | 45 | 55 | 5 | 95 | 40 | 60 | P.M. |
| 14 | 10 | Pond | 2 | 10 | 90 | 0 | 100 | 40 | 60 | A.M. |
| 15 | 40 | Pond | 8 | 37 | 63 | 20 | 80 | 18 | 82 | A.M. |
| 16 | 80 | Pond | 8 | 6 | 94 | 8 | 92 | 38 | 62 | A.M. |
| 17 | 40 | Pond | 8 | 47 | 53 | 10 | 90 | 15 | 85 | P.M. |
| 18 | 40 | Pond | 8 | 45 | 55 | 0 | 100 | 20 | 80 | P.M. |
| 19 | 28 | Pond | 4 | 53 | 47 | 14 | 86 | 29 | 71 | P.M. |
| Total | 571 | Av. . | 5.1 | 32.3 | 67.7 | 12 | 88 | 27 | 73 | |

In an infinite series of experiments, if the factors considered did not influence the movements, 25 per cent. should have been toward the water, 12.5 per cent. toward the sun and 12.5 per cent. toward the direction the head originally pointed. The

results obtained from the experiments were; 32.3 per cent. toward the water, 12 per cent. toward the sun, and 27 per cent. toward the direction the head originally pointed. The per cent of movements toward the water is too low to warrant the assumption that the movements of the group, as a whole, have been influenced by the water; however, certain individuals exhibit a marked tendency to move toward the water. One individual made 87 per cent. of movements toward the water; five, between 50 and 60 per cent.; three, between 40 and 50 per cent. Thus nine individuals, practically half of the individuals used in the experiments exhibited a tendency to move toward the water.

Brightness did not influence the movements. There was a slight tendency to move in the direction the head was originally pointed.

When these beetles were given their freedom, some flew and others did not. Of those that flew, more than 90 per cent. flew to the water.

### Small Diving Beetle (*Laccophilus* sp.?).

To test their fitness for work on the checkerboard plate, a few experiments were conducted with a small diving beetle. On account of its proneness to fly it is a difficult subject with which to work; but, where one has sufficent patience it may be used. It might be a good idea to clip its second pair of wings. On escaping they always flew toward the water.

### The Large Water Strider (*Gerris remigis* Say) (Plate VI).

In working with the checkerboard plate, 27 individuals were induced to perform 534 experiments. Seventeen individuals were used near the stream where they were captured; the remainder were taken to the shore of a lake. In most cases the board was placed from five to ten feet from the water. In a few cases it was necessary to place it twenty feet away. The results are recorded in Table VII.

In an infinite series of experiments, if the factors considered, have no directive influence on the animals, then 25 per cent. of the movements should be toward the water, 12.5 per cent. toward the sun and 12.5 per cent. toward the original position of the head. The experiments yielded 34.7 per cent. of movements

toward the water, 16 per cent. toward the sun and 26.6 per cent. toward the initial position of the head. The low per cent of movements toward the water is too low to warrant the conclusion that the group, as a whole, is directed, in its movements, by the nearest body of water. However, certain individuals are undoubtedly so influenced. Two individuals made 100

TABLE VII.

WATER-STRIDER (*Gerris remigis* Say).

| Specimen Number | Number of Trials | Age. | Kind of Water | Initial Positions. | Per Cent. of Movements. | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Toward Water | Not Toward Water | Toward Sun. | Not Toward Sun. | Way Head Pointed. | Not Way Head Pointed. |
| 1 | 10 | Adt. | Br. | 3 | 80 | 20 | 0 | 100 | 30 | 70 |
| 2 | 5 | Adt. | Br. | 4 | 40 | 60 | 0 | 100 | 40 | 60 |
| 3 | 1 | Juv. | Br. | 1 | 0 | 100 | 0 | 100 | 0 | 100 |
| 4 | 12 | Juv. | Br. | 8 | 33 | 67 | 1 | 99 | 50 | 50 |
| 5 | 2 | Adt. | Br. | 2 | 0 | 100 | 0 | 100 | 0 | 100 |
| 6 | 11 | Juv. | Br. | 5 | 9 | 91 | 0 | 100 | 27 | 73 |
| 7 | 11 | Adt. | Br. | 6 | 9 | 91 | 0 | 100 | 27 | 73 |
| 8 | 4 | Adt. | Br. | 1 | 25 | 75 | 50 | 50 | 0 | 100 |
| 9 | 2 | Adt. | Br. | 1 | 100 | 0 | 0 | 100 | 0 | 100 |
| 10 | 12 | Adt. | Br. | 3 | 0 | 100 | 50 | 50 | 0 | 100 |
| 11 | 2 | Adt. | Br. | 1 | 50 | 50 | 50 | 50 | 0 | 100 |
| 12 | 20 | Juv. | Br. | 4 | 30 | 70 | 25 | 75 | 50 | 50 |
| 13 | 1 | Juv. | Br. | 1 | 100 | 0 | 0 | 100 | 0 | 100 |
| 14 | 18 | Adt. | Br. | 3 | 11 | 89 | 89 | 11 | 17 | 83 |
| 15 | 9 | Adt. | Br. | 2 | 44 | 56 | 22 | 78 | 22 | 78 |
| 16 | 20 | Adt. | Br. | 4 | 20 | 80 | 50 | 50 | 50 | 50 |
| 17 | 6 | Adt. | Lake | 2 | 50 | 50 | 17 | 83 | 0 | 100 |
| 18 | 28 | Adt. | Lake | 6 | 36 | 64 | 14 | 86 | 61 | 39 |
| 19 | 40 | Adt. | Lake | 8 | 43 | 57 | 0 | 100 | 33 | 67 |
| 20 | 40 | Adt. | Lake | 8 | 25 | 75 | 7 | 93 | 52 | 48 |
| 21 | 40 | Adt. | Lake | 8 | 52 | 48 | 3 | 97 | 35 | 65 |
| 22 | 40 | Adt. | Lake | 8 | 18 | 82 | 2 | 98 | 40 | 60 |
| 23 | 40 | Adt. | Lake | 8 | 57 | 43 | 25 | 75 | 32 | 68 |
| 24 | 40 | Adt. | Lake | 8 | 32 | 68 | 5 | 95 | 40 | 60 |
| 25 | 40 | Adt. | Lake | 8 | 17 | 83 | 8 | 92 | 33 | 67 |
| 26 | 40 | Adt. | Lake | 8 | 30 | 70 | 7 | 93 | 42 | 58 |
| 27 | 40 | Adt. | Lake | 8 | 25 | 75 | 8 | 92 | 35 | 65 |
| Total | 534 | Average..... | | 5 | 34.7 | 65.3 | 16 | 84 | 26.6 | 73.4 |

per cent. of movements toward the water; one, 80 per cent; four, between 50 and 60 per cent. On the other hand it must be noted that three individuals made 100 per cent. of their movements

away from the water; three, between 90 and 100 per cent.; four, between 80 and 90 per cent.; five, between 70 and 80 per cent.; four, between 60 and 70 per cent.; four, between 50 and 60 per cent. Evidently, although some individuals are undoubtedly influenced by water as a directive force, this is certainly not true of the group as a whole.

Near a small pond in Carondelet Park, St. Louis, Mo., there is a narrow valley. The floor, which is nearly horizontal is about 60 feet long and 20 feet wide. About eight feet from the pond it slopes, at an angle of 60 degrees to the water. On the two sides the walls slope upward at an angle of from 30 to 50 degrees. This valley floor was selected as the place in which to perform the following experiments with water-striders which had been captured in a brook near Creve Coeur Lake, Mo. One at a time the water-striders were placed on the ground, at a certain definite distance from the water. They were always faced away from the water. They were watched continuously until they had either reached the water or had gone from the starting point, in some direction other than toward the water, a distance equal to that from the starting place to the water. Such individuals were recorded as not moving toward the water. Sometimes an individual would start in a certain direction and continue in that direction to the close of the experiment. More often it wandered about in various directions before settling down to continue in one course. Sometimes an individual would move toward the water a distance of ten to twenty feet and then turn about and move away from it. In a few cases an individual would move away from the water a distance of ten to fifteen feet and then turn, meander about, and finally reach the water. Several of those that did not reach the water climbed the sides of the valley for a distance of fifteen to twenty feet. If, in its wanderings, an individual happened to arrive on the steep slope that extended from the horizontal floor of the valley to the pond, it invariably continued, sometimes with accelerated speed, to the water. The results are recorded in Table VIII.

These results harmonize with the conclusions based upon experiments with the checkerboard plate.

TABLE VIII.

| Number of Individuals Used. | Number of Feet Placed From Water. | No. that Moved. | | Per Cent that Moved. | |
|---|---|---|---|---|---|
| | | To the Water. | Not Toward the Water. | To the Water. | Not Toward the Water. |
| 20 | 10 | 6 | 14 | 30 | 70 |
| 10 | 20 | 2 | 8 | 20 | 80 |
| 10 | 30 | 2 | 8 | 20 | 80 |

THE WHIRLIGIG BEETLE (*Gyrinus* sp.?) (PLATE VII).

In working with the checkerboard plate twenty-one individuals were used. With the board from ten to fifteen feet from the water, 551 experiments were performed. The results are recorded in Table IX.

In an infinite series of experiments, if the factors considered have no effect of the movements of the creatures, then 25 per cent. of the movements should have been toward water; 12.5 per cent., toward the sun; 12.5 per cent. toward the original position of the head. These experiments yielded 52.6 per cent. of movements toward the water, 6.7 per cent. toward the position of the sun and 22 per cent. toward the original position of the head. In one individual the per cent. of movements toward the water was 95, in four cases it was between 80 and 90, in three cases it was between 60 and 70, in four cases it was between 50 and 60, in five it was between 40 and 50. In the case of only three individuals was the per cent. of movements toward water less than 30. Evidently, the whirligig beetle, when moving on the checkerboard plate, is largely directed by the nearest body of water. They act as though they were positively hydrotropic.

In addition to the work upon the checkerboard plate, the following experiments were conducted in the open. Twelve specimens were placed in a dry tray ten feet from the water. In less than half an hour all had flown to the water. Ten specimens were placed in a dry tray twenty feet from the water. In less than half an hour all had flown to the water. Ten specimens

TABLE IX.

THE WHIRLIGIG BEETLE (*Gyrinus* sp. ?.)—ADULTS.

| Specimen Number. | Number of Trials. | Kind of Water. | Initial Positions. | Per Cent. of Final Movements. | | | | | | Time of Day. |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Toward Water. | Not Toward Water. | Toward Sun. | Not Toward Sun. | Way Head Pointed. | Not Way Head Pointed. | |
| 1 | 5 | Pond | 1 | 80 | 20 | 0 | 100 | 0 | 100 | A.M. |
| 2 | 35 | Pond | 7 | 60 | 40 | 6 | 94 | 6 | 94 | A.M. |
| 3 | 17 | Pond | 4 | 88 | 12 | 0 | 100 | 29 | 71 | A.M. |
| 4 | 2 | Pond | 1 | 0 | 100 | 0 | 100 | 100 | 0 | A.M. |
| 5 | 9 | Pond | 2 | 11 | 89 | 0 | 100 | 100 | 0 | A.M. |
| 6 | 5 | Pond | 1 | 0 | 100 | 0 | 100 | 20 | 80 | A.M. |
| 7 | 40 | River | 8 | 45 | 55 | 7 | 93 | 27 | 73 | A.M. |
| 8 | 5 | River | 1 | 40 | 60 | 0 | 100 | 0 | 100 | A.M. |
| 9 | 40 | River | 8 | 55 | 45 | 15 | 85 | 13 | 87 | A.M. |
| 10 | 17 | River | 3 | 37 | 63 | 5 | 95 | 12 | 88 | P.M. |
| 11 | 40 | River | 8 | 87 | 13 | 0 | 100 | 13 | 87 | A.M. |
| 12 | 41 | River | 8 | 56 | 44 | 0 | 100 | 20 | 80 | A.M. |
| 13 | 13 | River | 3 | 77 | 23 | 0 | 100 | 8 | 92 | A.M. |
| 14 | 40 | River | 8 | 30 | 70 | 15 | 85 | 15 | 85 | A.M. |
| 15 | 40 | River | 8 | 88 | 12 | 3 | 97 | 0 | 100 | P.M. |
| 16 | 41 | River | 8 | 68 | 32 | 30 | 70 | 17 | 83 | P.M. |
| 17 | 41 | River | 8 | 58 | 42 | 2 | 98 | 36 | 64 | P.M. |
| 18 | 40 | River | 8 | 43 | 57 | 15 | 85 | 15 | 85 | P.M. |
| 19 | 40 | River | 8 | 42 | 58 | 13 | 87 | 7 | 93 | P.M. |
| 20 | 20 | River | 4 | 95 | 5 | 0 | 100 | 20 | 80 | P.M. |
| 21 | 20 | River | 4 | 45 | 55 | 30 | 70 | 5 | 95 | P.M. |
| Total | 551 | Av. | 5.2 | 52.0 | 47.4 | 6.7 | 93.3 | 22 | 78 | |

were placed in a dry tray forty feet from the water. In less than half an hour all had flown to the water. In most cases they did not ascend high in the air; but the further back they were the higher they ascended. In one case a sidewise flight carried the beetle behind a tall tree. In that case it arose to above the top of the tree before turning toward the water.

At 3:15 P.M. the eyes of ten individuals were blinded by painting them with lampblack mixed in liquid glue. These whirligigs were placed in a dry tray forty feet from the water. Ten normal individuals were placed in a similar tray, at the same distance from the water. The two trays were only two feet apart. One by one, the individuals with normal sight arose from the tray and flew to the water. By 3:30 P.M. all but one had reached the water. At 4:00 P.M. the last one flew to the water. Up to

4:15 P.M., when I stopped for the day, none of the blinded whirligigs had reached the water. Two had become lost in the grass. Eight were still in the tray.

These experiments show conclusively that the nearest body of water exerts a directive influence upon the movements of the whirligig beetle and that the control is exerted by means of stimulus received through the eyes.

## Conclusions

1. The checkerboard plate consists of a smooth board covered with white oilcloth which is subdivided, by printed lines, into one-inch squares. There is a leveling device by means of which it can be made perfectly horizontal. Considering the center of the board the center of a circle, the board is divided into eight sections, each forty-five degrees wide. Starting at the middle of one side the tips of these dividing lines are numbered from 0 to 315. This nomenclature makes it possible to arrange the specimen at the center of the board, with its head pointing in a certain definite direction. When in use, by means of an adjustable screen, the board is protected from the direct rays of the sun. It is washed from time to time to free it from odors that may influence movements. Thus manipulated all possibility of geotactic, chemotactic, and thigmotactic responses are eliminated; hence this device forms an excellent means of investigating the influence of bodies of water upon the behavior of small animals.

1. It may be used in studying any walking land insect, milliped, centiped, crustacean, or annelid.

2. It may be used in studying flying insects that also walk; but, in some cases it may be necessary to clip the wings.

3. It may be used in investigating the behavior of many fresh-water invertebrates (small snails, amphipods, isopods, crayfish, some backswimmers, giant water bugs, water-striders, larvæ of straight-winged flies, dragonfly larvæ, scavenger beetles, diving beetles, whirligig beetles); in some cases it cannot be used because the animals cannot remain out of water long enough (damsel-fly larvæ); in yet others it cannot be used because the creatures are too awkward when out of the water (large snails, water-tigers).

4. Although the matter has not been tested, it should be possible to use it in investigating many marine invertebrates.

5. This device may be used, not only with creatures with sound vision; but, also, with forms that have been rendered blind by manipulation.

II. In testing this device several ecological types of invertebrates were used: those that creep along the bottom of the body of water or along water plants, but do not leave the pond or stream (*Asellus*, half grown dragonfly larvæ, pond snails [these occasionally venture beyond the water edge]); those that creep along the bottom or up vegetation and occasionally leave the water (crayfish, scavenger beetles); those that live on the film and never depart far from it (giant water striders); those that dwell on the film, but migrate from one body of water to another (whirligig beetles).

1. The movements of neither pond snails, asellids nor dragonfly larvæ are influenced by nearby bodies of water.

2. In the majority of cases the movements of crayfish are controlled by the nearest body of water. When out of the water they behave as though they are positively hydrotropic. Blind crayfish do not so react; this demonstrates that the movements of this crustacean, when out of the water, are controlled by visual stimuli furnished by the nearest body of water.

3. About half of the scavenger beetles investigated act as though they are positively hydrotropic; the other half are not influenced, in a directive manner, by nearby bodies of water.

4. When on land, the majority of the water striders investigated are not influenced, in their movements, by the nearest body of water; a few act as though they are positively hydrotropic.

5. When out of the water, whirligig beetles are undoubtedly influenced, in their movements, by the nearest body of water. On the checkerboard plate the majority always move toward the water; when set at liberty within forty feet of a body of water, they always fly toward it. If blinded they do not react in this manner. This demonstrates that they are controlled by visual stimuli furnished by the water.

6. This paper does not contend that these movements which some of the forms make toward water are positive hydrotropisms

in the Loebian sense. These investigations prove that the movements mentioned are caused by the directive power of some body of water and not by gravity, nor the sun's rays, nor contact stimuli, nor odor trails. As to whether they are responses to sensations or are merely tropisms it has nothing to say. Either interpretation seems equally plausible.

7. Although the board was protected from the direct rays of the sun, almost all of the species used exhibited a marked tendency to move away from that portion of the board which was nearest the position of the sun. Since the sun's rays did not impinge on the individuals, this avoidance cannot be a negative phototropism. It must be a form of differential sensitivity. Perhaps it should be called negative photosensitivity.

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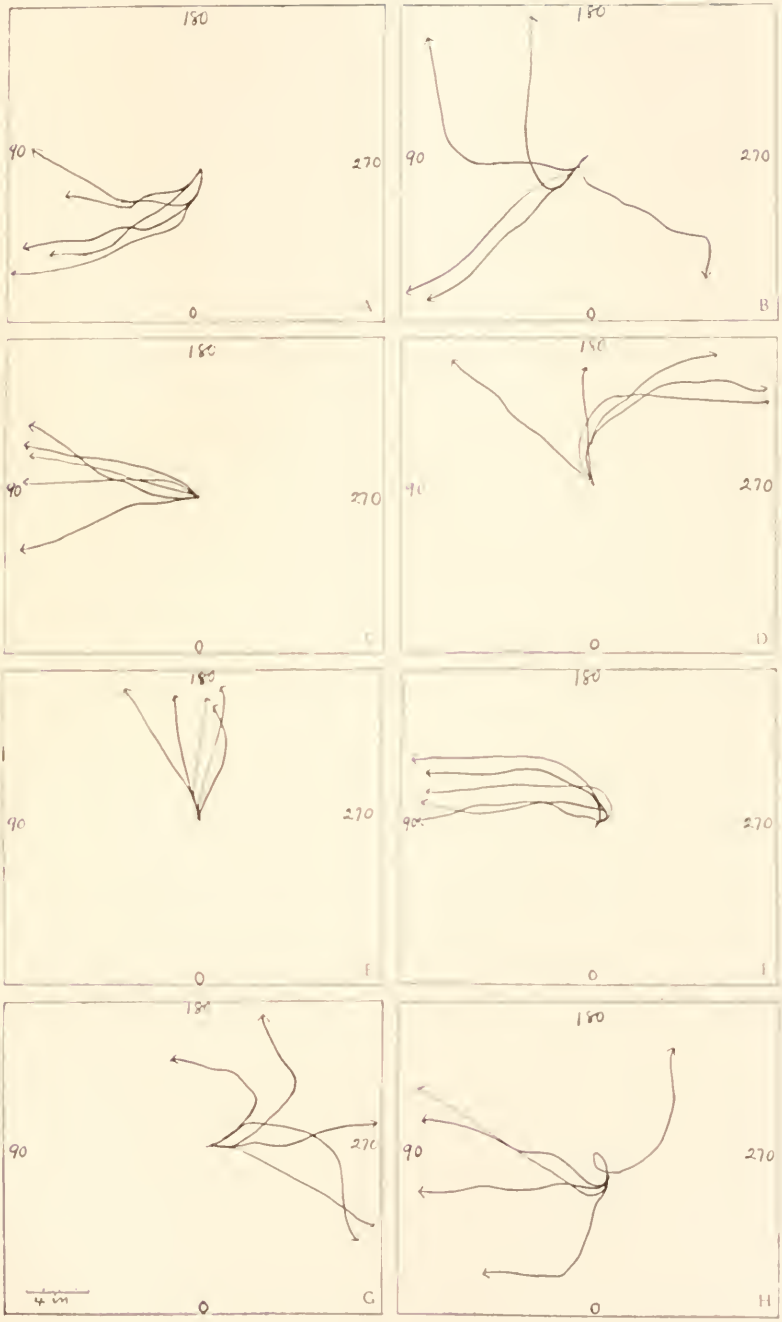


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PLATE I. DRAGON FLY NYMPHS, SERIES OF 40 TRIALS.

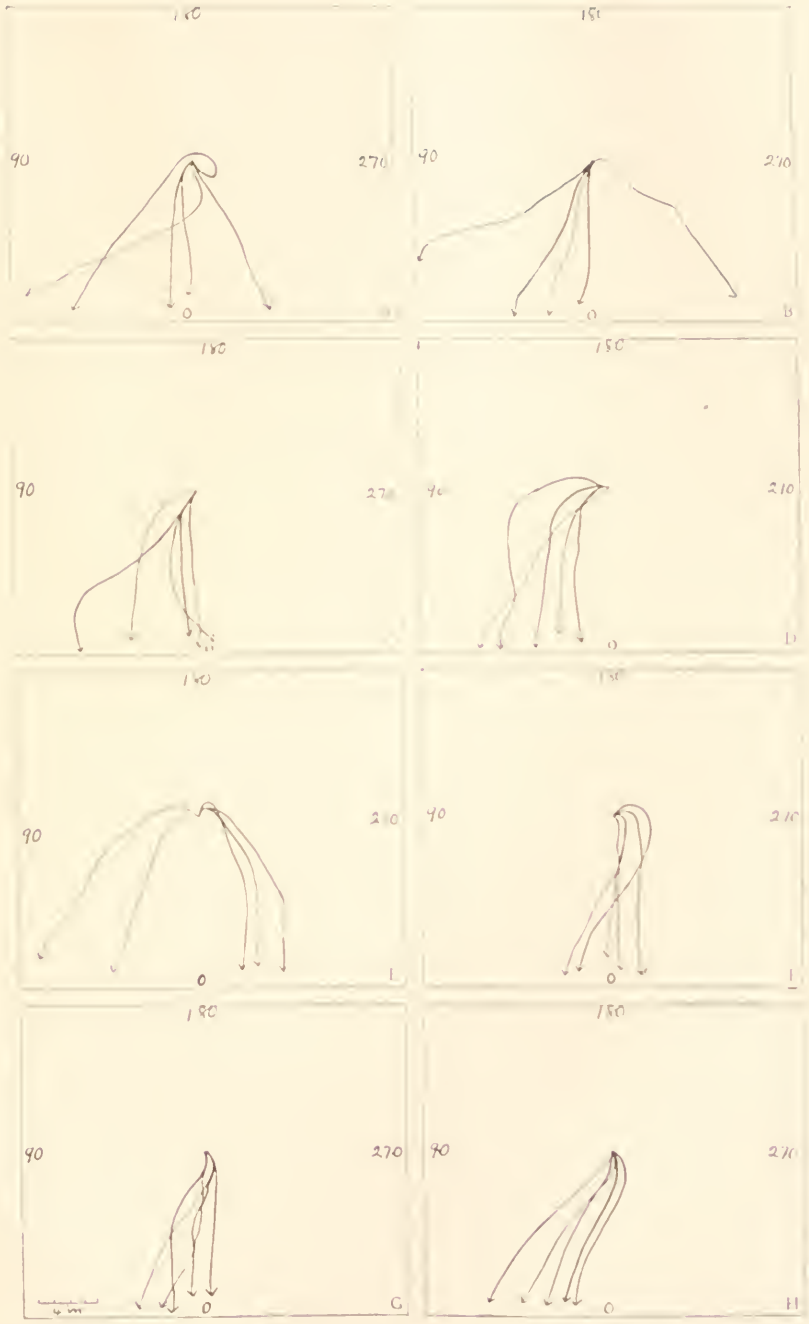


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PLATE II. NORMAL CRAYFISH.

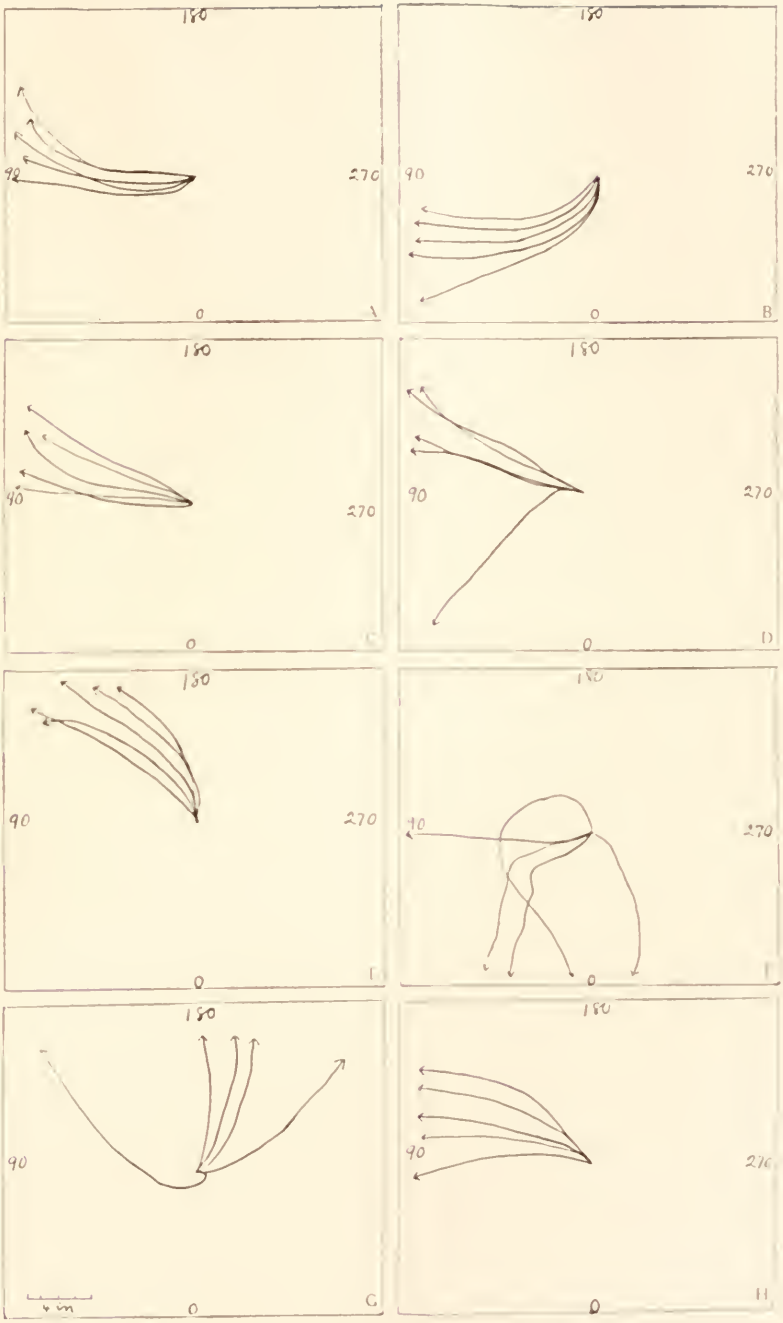


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PLATE III. BLINDED CRAYFISH.

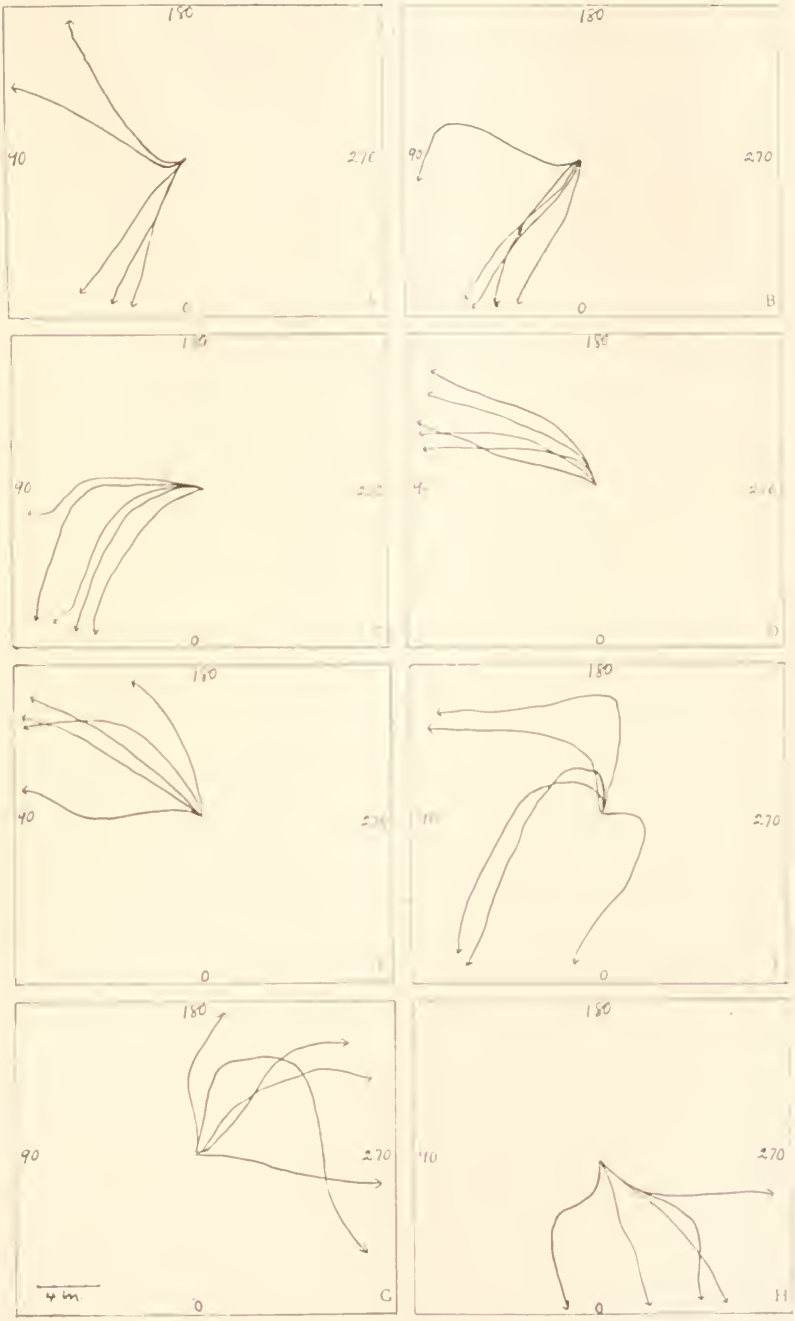


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PLATE IV. GIANT WATER BUG.

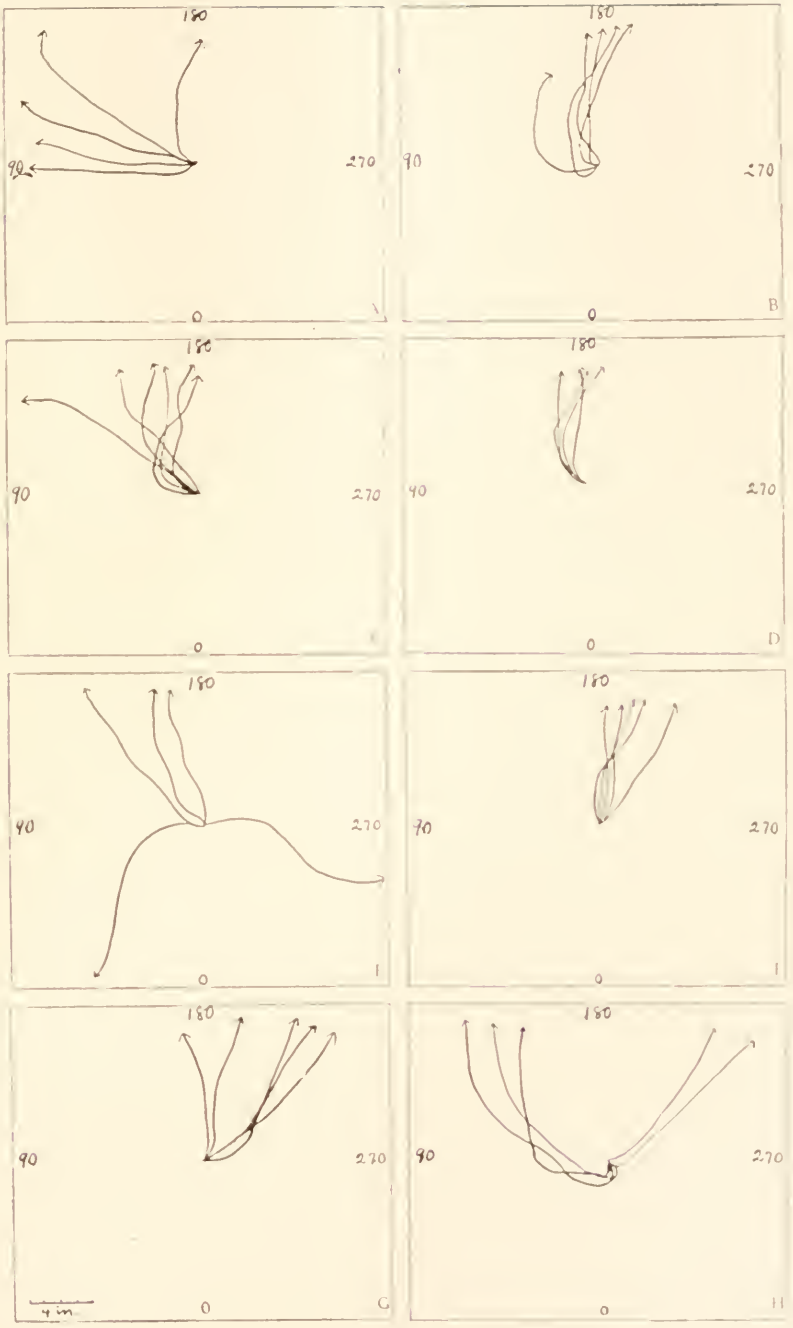


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PLATE V. SCAVENGER BEETLE.

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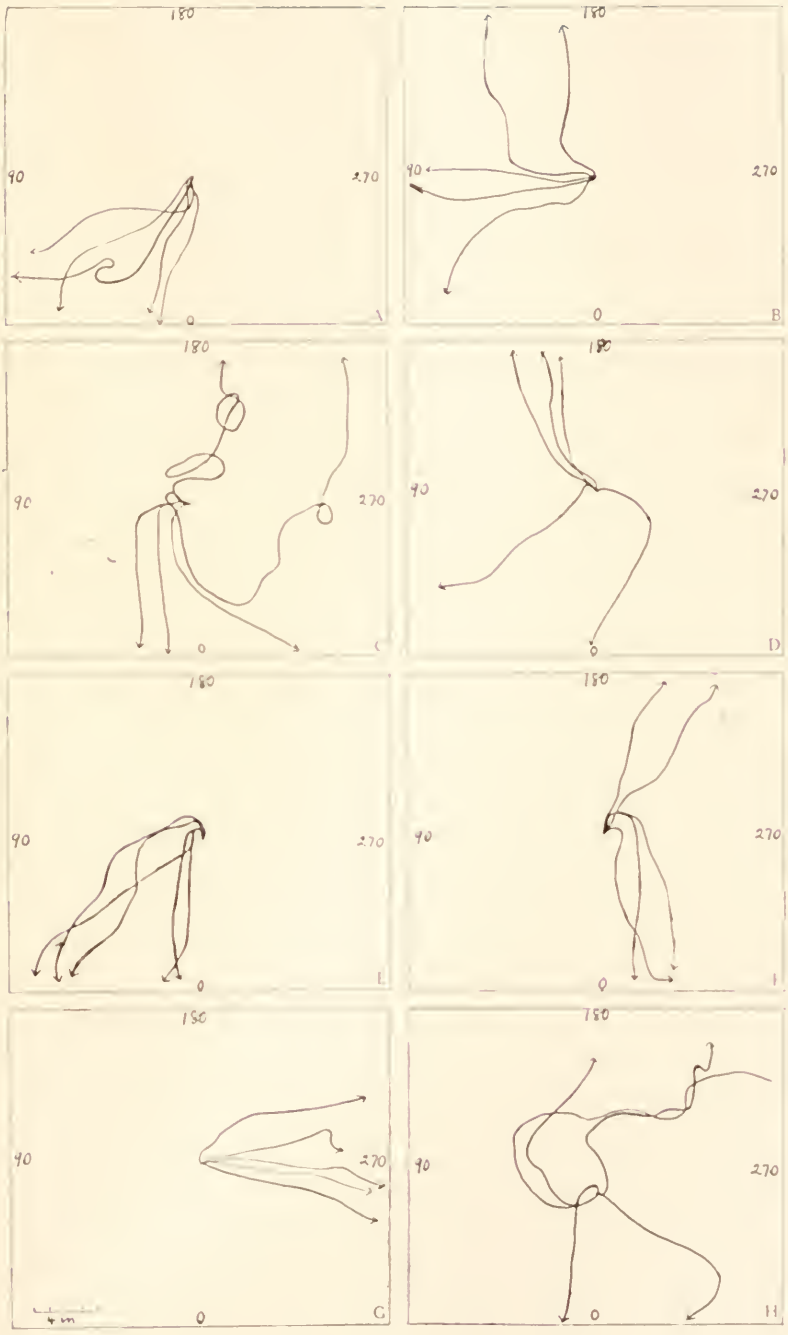
PLATE VI. LARGE WATER STRIDER.




C. H. TURNER.

PLATE VII. THE WHIRLIGIG BEETLE.

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# BIOLOGICAL BULLETIN

## MODIFICATION OF RESPONSE IN AMŒBA.

S. O. MAST AND L. C. PUSCH.

From the Zoölogical Laboratory of the Johns Hopkins University.

Gibbs and Dellinger ('08) and Schaeffer ('17) maintain that Amœba has the power of selecting food. The former hold that the kind of food preferred changes under various conditions, and they suggest that changes in the kind of food selected is the result of a process of learning based upon the method of "trial and error." One of us (Mast '10) in preceding observations found that when the tip of a pseudopod of an amœba comes in contact with a highly illuminated region in the field it stops; then others are sent out in succession in the same general direction, each one stopping when it comes in contact with the light, until suddenly one is sent out in a markedly different direction, avoiding the illuminated region altogether. It is maintained that this abrupt change in the direction of the formation of the pseudopods, constituting a profound modification in the response to a given stimulus, is dependent upon the preceding experience of the individual, *i.e.* repeated contact with the strong light.

The specimens used in the following experiments were obtained from the cultures in the laboratory, most of which had been stocked with material from a stream near the campus. They were of the proteus type. Each amœba was isolated on a glass slide in a drop of its culture-fluid under a cover-glass, the edges of which were sealed with vaseline. Each amœba was adapted to darkness before using.

The observations were made in the dark room. An area of intense light was obtained by focusing on the slide under the microscope, by means of the reflector and an Abbe condenser, an image of a portion of the luminous filament of an incandescent Mazda lamp placed in a light-tight box. The rays of the filament passed through a very narrow slit in the box. The image had

clearly defined edges and furnished a band of intense light across the field. The slide was adjusted so that the amœba was within a distance equal to approximately half its length from the light with the pseudopod or pseudopods progressing towards it. If the pseudopod did not stop when it came in contact with the light the amœba was discarded. If it stopped the behavior of the amœba was observed until it definitely moved away from the light, and the number of pseudopods which made contact with it was recorded. After this the light was shut off, and the amœba was left in darkness until the next trial was made. In this way each individual was given twenty-seven trials.

The interval between trials was approximately three minutes except in the following as recorded in Table I: individual *B*, trials 16–17 and 23–24, 24 hours; individual *D*, trials 15–16, 22 hours; individual *E*, trials 8–9 18 hours, 16–17, 48 hours, and 21–22, 24 hours; individual *F*, trials 8–9, 50 hours, and 20–21, 20 hours; individual *G*, trials 16–17, 16 hours, and 24–25, 3 hours. During these intervals the amœbæ were kept on their slides in darkness undisturbed.

The behavior of a typical individual is illustrated in Fig. 1 by sketches representing all of the trials in the series. By referring to this figure it will be seen that while during the first part of the series there was something of a persistence on the part of the amœba to attempt to proceed in its original direction—notably 7 attempts in trial 4—there was but little in the latter part of the series—a total of only 5 attempts during the last 14 trials.

The results obtained in all of the tests are presented in Table I. This table shows that the number of pseudopods which came in contact with the light before the direction was changed decreased in general as the number of trials increased in each series, that there was a similar decrease in the total contacts of all amœbæ for corresponding trials, and also in the totals of consecutive groups of three trials. Amœba *B* made 10 contacts with the light in the first three trials; with trial 4 a general decrease begins. A total of 20 pseudopods made contact in the first 14 trials of the series, and a total of 9 in the last 13. In the series of amœba *D* there were 5 contacts in the first 6 trials but none at all in the remaining 21 trials. Amœba *E* made 21 contacts in the first 14 trials—11 of these were in the first 4—and 5 in the last 13

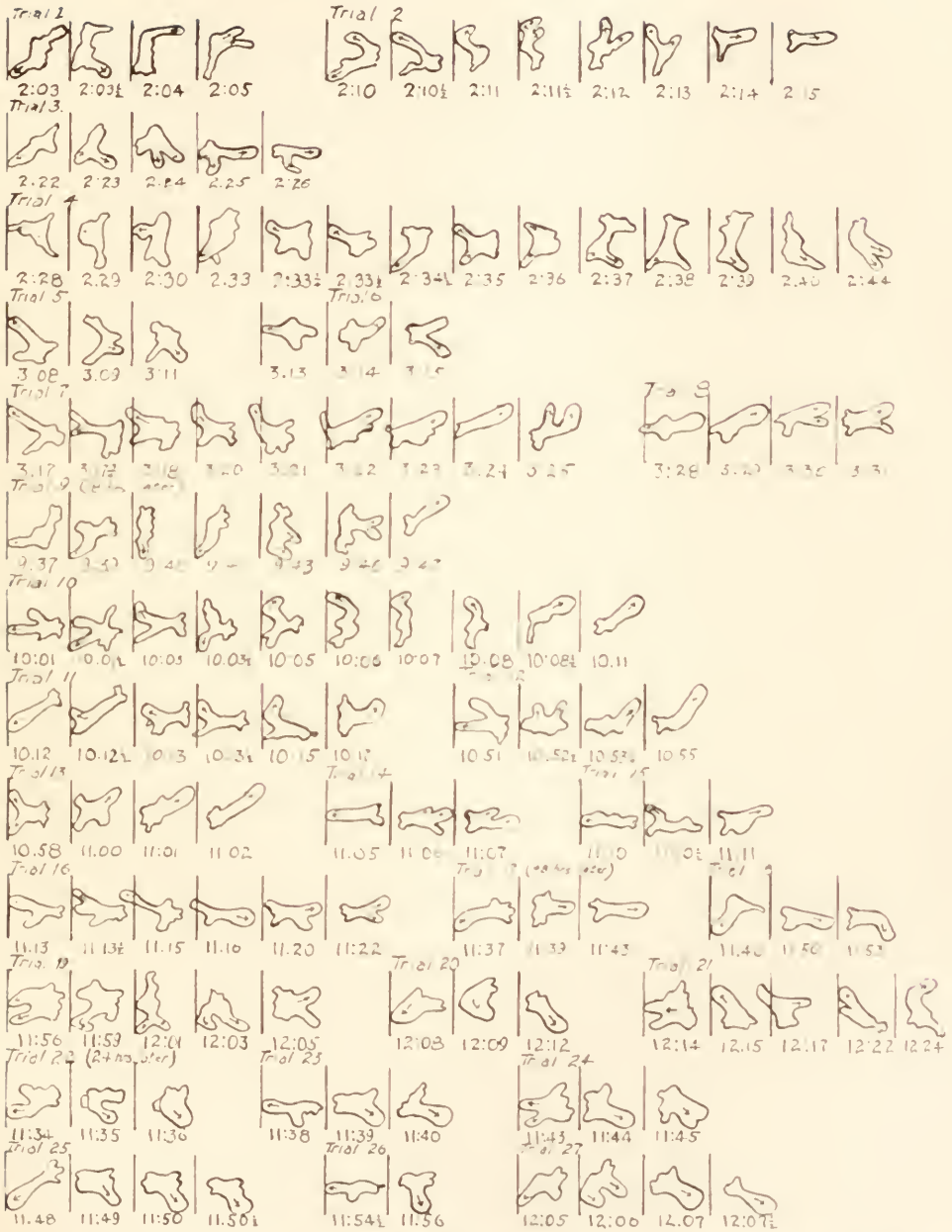


FIG. 1. A series of diagrammatic sketches illustrating the behavior of Amœba *E* for twenty-seven trials. The straight lines of equal length represent a band of light. The outlines of the amœba describe its position in relation to the light at the time given below in each individual sketch. The arrows show the direction of protoplasmic streaming.

trials. Amœba *F* made no contacts until trial 8; between trials 8 and 15 there were 8 contacts, while in the remaining 12 trials there was but one contact. There was one contact in the first trial of Amœba *G*, then none until the 7th, when a reversal began and persisted very generally through the 17th, by which time 23 contacts had been made; in the remaining ten trials there were 8 contacts.

TABLE I.

RESPONSE OF AMŒBA TO CONTACT WITH A HIGHLY ILLUMINATED REGION IN THE FIELD.

| Designation of Trials. | Number of Pseudopoda that Came in Contact with Light in Each Trial. | | | | | Total Contacts for All Individuals in Each Trial. | Total Contacts for All Individuals in Groups of Three Consecutive Trials. |
|---|---|---|---|---|---|---|---|
| | *B.* | *D.* | *E.* | *F.* | *G.* | | |
| 1 | 4 | 0 | 0 | 0 | 1 | 5 | |
| 2 | 3 | 0 | 3 | 0 | 0 | 6 | |
| 3 | 3 | 3 | 1 | 0 | 0 | 7 | 18 |
| 4 | 0 | 0 | 7 | 0 | 0 | 7 | |
| 5 | 3 | 1 | 0 | 0 | 0 | 4 | |
| 6 | 0 | 1 | 0 | 0 | 0 | 1 | 13 |
| 7 | 1 | 0 | 2 | 0 | 1 | 4 | |
| 8 | 2 | 0 | 0 | 2 | 4 | 8 | |
| 9 | 0 | 0 | 2 | 1 | 2 | 5 | 17 |
| 10 | 0 | 0 | 4 | 0 | 1 | 5 | |
| 11 | 3 | 0 | 2 | 2 | 2 | 9 | |
| 12 | 0 | 0 | 0 | 0 | 0 | 0 | 14 |
| 13 | 0 | 0 | 0 | 1 | 2 | 3 | |
| 14 | 1 | 0 | 0 | 0 | 4 | 5 | |
| 15 | 0 | 0 | 1 | 2 | 1 | 4 | 12 |
| 16 | 1 | 0 | 1 | 0 | 1 | 3 | |
| 17 | 0 | 0 | 0 | 0 | 5 | 5 | |
| 18 | 0 | 0 | 0 | 0 | 0 | 0 | 8 |
| 19 | 0 | 0 | 1 | 0 | 0 | 1 | |
| 20 | 0 | 0 | 0 | 0 | 1 | 1 | |
| 21 | 2 | 0 | 2 | 0 | 0 | 4 | 6 |
| 22 | 0 | 0 | 0 | 1 | 0 | 1 | |
| 23 | 1 | 0 | 0 | 0 | 3 | 4 | |
| 24 | 0 | 0 | 0 | 0 | 3 | 3 | 8 |
| 25 | 4 | 0 | 0 | 0 | 1 | 5 | |
| 26 | 1 | 0 | 0 | 0 | 0 | 1 | |
| 27 | 0 | 0 | 0 | 0 | 0 | 0 | 6 |

The totals by groups of three consecutive trials fluctuate between 18 for the first group, 12 for the middle and 6 for the last. In other words there is a modification of response in the sense that the number of attempts to continue in the original direction after meeting the light decreases in general as the number of trials of the series increases.

It will be remembered that each series was interrupted irregularly by longer intervals of time, usually about a day or longer. Despite this there is a general constancy of the tendency throughout each series. This seems to indicate ability to retian for some time the modification of response.

These experiments were repeated 18 months after the preceding portion of the paper was completed. Two individuals were used and a series of 18 readings approximately 3 minutes apart were obtained with each. The total number of contacts made by these two individuals in groups of three consecutive trials beginning with the first follows: 20, 9, 10, 6, 7, 4. The results obtained in these observations are consequently in harmony with those obtained in the preceding observations.

## Summary.

When Amœba repeatedly comes in contact with a band of intense light the number of attempts to continue in the original direction decreases as the number of trials increases.

This indicates that there is some change in Amœba that is analogous to what is called "learning" in the higher animals.

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# AN EXPERIMENTAL STUDY AND A PHYSIOLOGICAL INTERPRETATION OF EXOGASTRULATION AND RELATED MODIFICATIONS IN ECHINODERM EMBRYOS.

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## Introductory.

In a study of the means of altering and controlling the course of development in echinids by electrolytes the writer had occasion to note the action of lithium chloride in effecting remarkable form changes in the early ontogeny of sand-dollars and sea-urchins. An attempt was then made to analyze further these effects and the similar ones so extensively studied by Herbst and others, and to provide for the wealth of facts obtained some more consistent and satisfactory explanation than has been offered.

Application to these cases of the susceptibility and differential inhibition methods of Child has supplied what seems a secure and reasonable basis for interpreting such puzzling teratological forms as exogastrulæ, "holentoblastulæ," etc. For I believe that the first impression of an exquisitely specific action of the $Li'$ ion is not sustained by a closer examination of the facts, and that "lithium larvæ" fall logically into the series of abnormal forms producible quite at will by other means.

The work on *Echinarachnius*, *Arbacia*, and *Asterias* was carried out at Woods Hole in the summer of 1916 and that on *Strongylocentrotus* and *Orthasterias* at Friday Harbor in 1917. For materials and facilities at these stations the author is indebted to Dr. F. R. Lillie and Dr. W. C. Frye, and for many suggestions, discussions, and even for generous contribution of experimental data special thanks are extended to Dr. C. M. Child.

## Experimental.

*Physiological Axial Gradients.*—In scores of species of plants and animals of all degrees of complexity of organization a gradient

of metabolic activity and of other associated properties and conditions ($CO_2$ production, $O_2$ consumption, electrical potential, vital staining capacity, etc.) has been demonstrated by the help of many different methods (Child, '15*b* '20). Of these methods that of determining the relative susceptibility of various levels along the structural axis is most readily applicable in embryos, and has been found a generally reliable, if somewhat gross, indicator of the presence and nature of the physiological gradients, the significance of which for development has been emphasized by this writer.

*Methods.*—As applied to ontogenetic studies there are really *two interrelated susceptibility methods:* (*a*) direct determination of susceptibility of different parts of the egg or embryo; and (*b*) differential inhibition, differential acclimation and differential recovery in development.

(*a*) Agents in concentrations determined to be lethal within a few hours commonly cause death and disorganization first of the parts of the egg or embryo with most rapid metabolic activity and then later attack in succession in the same way those portions with progressively lower rates of metabolism—the susceptibility paralleling roughly the metabolic gradient (direct susceptibility). In lower concentrations which kill after a day or more the order of susceptibility was found to be reversed, since regions with more active metabolism acclimate more rapidly and completely (indirect susceptibility). For these purposes a vast number of lethal agents have been employed with generally concordant results, but the more toxic agents, such as cyanides, anesthetics, salts of heavy metals, basic dyes, etc., usually give best results; and with some of these tests were made on the direct susceptibility of the lithium-modified as compared with normal embryos (p. 73).

(*b*) When developmental stages are treated with the same agents in partially lethal concentrations or sublethal concentrations permitting acclimation or tolerance, or are returned to the normal medium after temporary exposure, a differential effect on development results. That is, the levels of highest metabolic rate in any gradient are more retarded or inhibited, or in case of acclimation or recovery, acclimate or recover more rapidly or more completely than levels of lower rate. In differential in-

hibition then the levels of lower rate may be relatively accelerated and in differential acclimation or recovery the levels of higher rate may be relatively accelerated after the primary inhibition. Direct differential acceleration is also possible under proper conditions. These differential effects of external agents in relation to the physiological gradients provide means of controlling to a greater or less extent the course of development and the sizes, proportions and relations of parts under given conditions (Child, '16, '17; Bellamy, '19).

In short, the established gradient of the egg or embryo, being physiological, is not necessarily permanently fixed and unalterable, but may be radically modified, obliterated entirely (as in *Asterias* embryos by N/1000 HCl in sea water), or even entirely reversed in direction, as we shall show for the lithium larvæ (Fig. 13).

The ordinary procedure in the differential inhibition method was as follows: Eggs of freshly collected animals were removed to a bowl, fertilized, and then placed during early cleavages in small quantities in liter Erlenmeyer flasks of sea water to which the agent used had been added. The flasks were loosely covered but not aërated. Each day the solution was renewed and embryos examined and notes and sketches taken recording the course of their development as compared with the controls kept in pure sea water. In most experiments some of the embryos were also removed after various times of exposure to the salt solutions to determine the possibility and nature of the steps of their recovery.

*The Lithium Modifications.*—Best results with lithium are contingent upon recognition of the prominent rôles of several factors; especially concentration of the agent, duration of exposure, and stage of development at time of exposure. In higher concentrations development is strongly inhibited and incomplete. If the concentration is too low, or if exposure is too brief, begins too late or is terminated at too early a stage, the effect is weak and development approaches by all transitions towards the normal. Herbst observed ('95*a*, ch. 3) that typical effects are not produced by temporary treatment merely up to fertilization, cleavage or young blastula stages; but well developed blastulæ treated with rather concentrated solutions and then returned to

sea water do show some of the lithium modifications much obscured by recovery. In general it may be said that maximal effects may be secured by sea water solutions of about N/60 to N/200 LiCl (according to the species of the embryo used) acting for a sensitive determining period from cleavage continuously through late blastula stages.

*Echinarachnius parma.*—This sand-dollar provides perhaps the most responsive material yet found for the study of lithium effects, which are here, if anything more diverse and extreme than in sea-urchins and far more so than in the star fishes used.

The action of lithium solutions producing the characteristic lithium effects is such as to retard or inhibit development, the degree of inhibition (and loss of power of recovery) varying directly with concentration. LiCl, 2 $H_2O$, N/60 or more, in sea water stops cleavage early; and even at N/80 only a few individuals reach the blastula stage. These survivors are either partial blastulæ or very small, immobile, thick-walled stereo-blastulæ, whose remarkably small segmentation cavity is packed so full of precociously formed opaque mesenchyme that much of the latter is forcibly extruded from the basal pole; these types, though not "sickly," die shortly without gastrulation. In N/100 death occurs most commonly in late cleavage, but one finds, inactive at the bottom of the container, a few stereo-blastulæ, which die as such, or, rarely, after assuming the constriction form described below.

In N/125 LiCl a considerable minority of the eggs become opaque mesenchyme-filled blastulæ, the larger of which, especially in more dilute (N/150) LiCl, often show a wide zone of thickening of the basal pole and after some delay begin the elongation preluding gastrulation; but the entoderm then evaginates after the fashion of that in lithium exogastrulæ. There is much variation in the diameter of the ring (usually basal) where the ectodermal (gastrula wall) and entodermal (archenteron) portions of the embryo join, but this ring is always enlarged—often so much so that it moves toward, to, or even past the equator of the blastula (Fig. 1, *n-q*); in the extreme cases the exogastrulation process appears to be merely a constriction between the animal and vegetal poles, forming two vesicles, one consisting

of the gastrula wall and the other of the gut entoderm. These two components may be of very different relative size (Fig. 2)

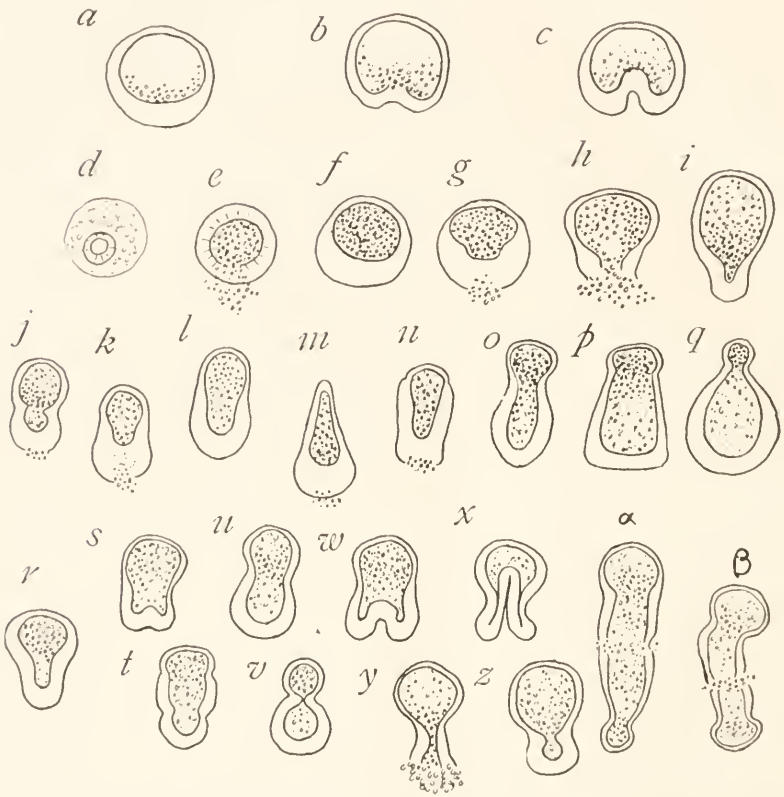


FIG. 1. Blastulæ and early gastrulæ of the Atlantic sand-dollar, *Echinarachnius parma*. *a–c*, normal embryos; others, typical embryos as modified by lithium. In the lithium series note: the overdevelopment and frequent basal extrusion of the mesenchyme; the various degrees of enlargement of the sharply defined, thick-walled endodermal portion; and the occasional occurrence (*s*, *w*, *x*) of partial endogastrulation. $\alpha$ and $\beta$ are double exogastrulæ, two joined terminally by their basal ends.

as might be expected from the mode of their formation. Sometimes the ectodermal portion exceeds the entodermal, often they are more or less equal, but in a great many the entoderm is clearly much the larger. In the last mentioned cases the ectodermal component becomes less and less, and approaches the vanishing point—a little knot of cells or nothing at all!—while the archenteron undergoes a compensatory and reciprocal hy-

pertrophy, until in a few individuals by its progressive enlargement the whole gastrula is entodermal (*i.e.* a "holentoblastula," better called for its physiological equivalent a holentogastrula).

There is never any real difficulty in distinguishing ectoderm and entoderm in these forms. In every species there are characteristic differences in pigmentation, in thickness of walls, in structure of the cells, in cilia, in size of cells, in frequency of

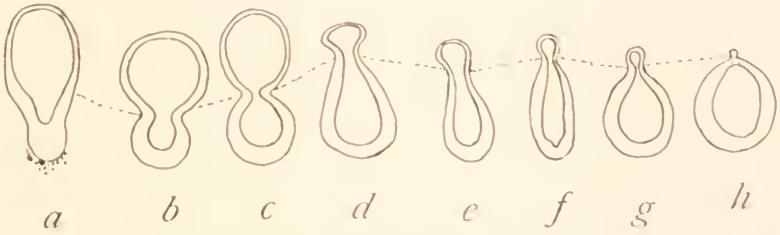


FIG. 2. Series of sand-dollar embryos (constriction forms) developed 27 hours in M/160 LiCl. Ectoderm (above) separated at dashed line from endoderm. Mesenchyme packing the blastocoels omitted. The ectodermal (thin-walled) component, larger in *a–c*, is reduced in size in *d–f*, and nearly obliterated in *g*, which is practically a holentogastrula.

nuclei, etc. From a study of the lineage of cells from the pigmented and nonpigmented zones of the early egg it may be stated quite positively that entoderm grows at the expense of ectoderm, some of which becomes converted into entoderm (and possibly also into mesenchyme).

Left in the solution many of these constriction forms show early death and gradual disintegration, sometimes of the apical ectoderm, or of both extremes, but usually of the entodermal component from its free end towards the attached part. Returned to sea water the more resistant ectodermal portion may recover and linger on awhile as a partial form, a holectogastrula as it were. This type may arise also in another way: if the constricted gastrulæ are removed early from the lithium and allowed to recover in sea water ectoderm and entoderm tend to separate from each other, especially when the two components are of nearly equal size (Fig. 3, *v*). It would seem that equality and duality are inconsistent with development of one individuality!

N/160 is close to the optimal concentration for producing the most striking lithium effects in this species. Retardation of

development is less general and more localized and differential. The eggs produce practically 100 per cent. modified blastulæ, which are smaller and thicker walled than their controls; the ectoderm does not thin out for some time even though the blastocœl is almost invariably solidly filled with mesenchyme. Such overproduction of mesenchyme seems to lead to a basal extrusion and loss of excess proliferated primary and secondary mesenchymal cells from the basal pole of the blastula, which is commonly

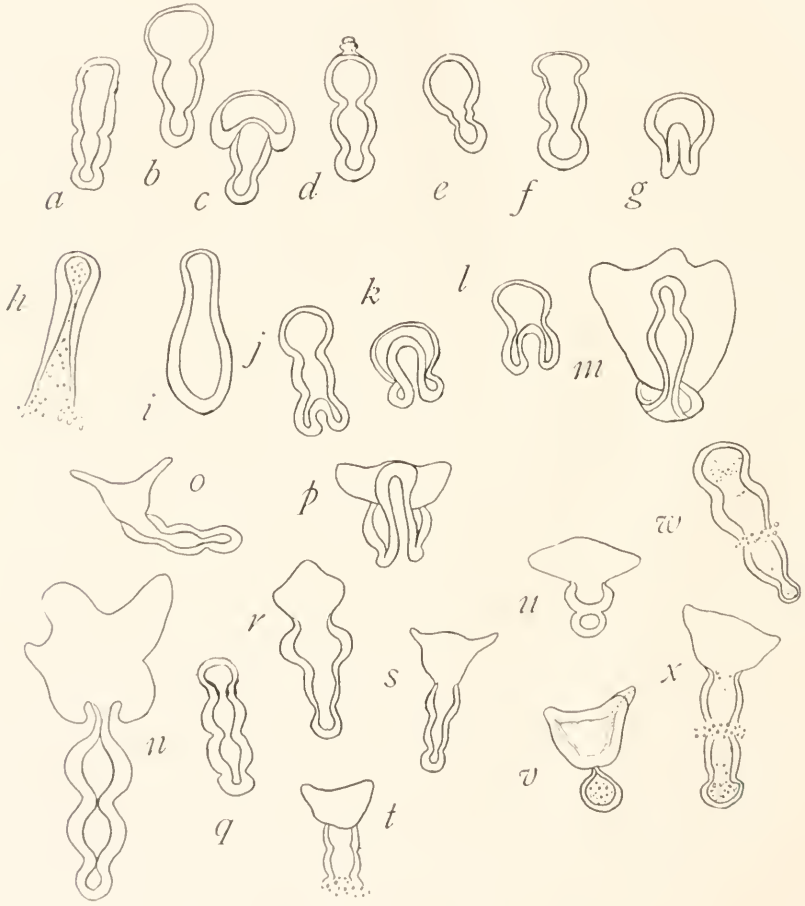


FIG. 3. Commonest types of sand-dollar gastrulæ and larvæ from lithium cultures. Show the wide range of lithium alterations. (See also other examples in the originals of Fig. 12). *a–i*, from M/160 and *j–x* from M/200 LiCl, returned at about 2 days to sea water. Mesenchyme omitted in most.

marked by the "cell rosette" of Herbst, composed sometimes of isolated cells and sometimes of loose masses, as if the blastula wall had actually burst at its weakest point through distention of its cavity. Shortly the entoderm begins to evert, all stages of exogastrulation are present (Fig. 3), and soon the culture consists almost entirely of exogastrulæ with only a few forms approaching normal endogastrulation. And here again one finds the whole gamut of types with regard to the relative size of the ectodermal and entodermal portions. The ectoderm shows all degrees of reduction down to complete obliteration in the large, relatively clear, holentogastrulæ, whose walls and contents exhibit all the distinguishing features of the archenteron. Nothing is more evident than that the entoderm grows here at the expense of the ectoderm.

At 33–36 hours or more those types in which both components are present may show further differentiation: the archenteron shows constrictions separating it into two, or its usual three divisions. This tri-partite gut is safely homologized with fore-, mid-, and hind-gut, with the fore-gut of course, situated at the most posterior free end of the embryo. The relative size of these three divisions shows a most interesting series of variations (Fig. 4). The total gut is greatly increased in bulk over the

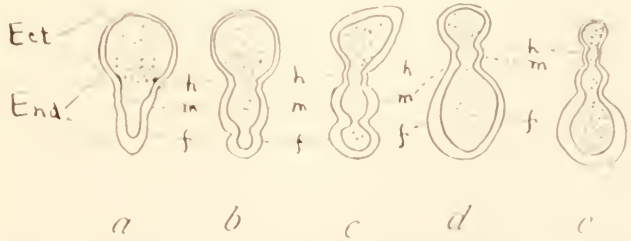


FIG. 4. Selected series of types from sand-dollar cultures developed in LiCl, M/150 to M/200. Showing, *a–e*, the wide range of reductions in the ectodermal portion (*ect.*); and the parallel changes of proportion of the endoderm (*end.*) in its three divisions: hind-gut (*h*); mid-gut (*m*); and fore-gut (*f*).

normal and could not conceivably be enclosed in the cavity of the ectoderm; and in this increase the fore-gut takes the greatest share, the mid-gut next, and the hind-gut (attached to the ectoderm) the least. There is an obvious and significant parallel in this process to the relative increase of the entoderm as a whole at the expense of reduction of the ectoderm.

Herbst demonstrated quite conclusively ('95 *a*, *e.g.* Figs. 17, 18, 22) that the thick entodermal layer of the lithium larvæ and of the intermediate types, is rich in nuclei and in the number of its smaller cells in proportion to the size of the part. The ectoderm, on the other hand, gives every evidence of retarded activity: at first very thick, it afterward becomes thin and stretched but is never composed of more than a single cell layer (except when and where the ciliary bands appear), and this layer is peculiarly sparse in nuclei and is composed of a few extraordinarily large cells, both in comparison with the normal ectoderm and with the entoderm of lithium larvæ.

The tip of the fore-gut often shows some disintegration, and the resulting cellular debris and associated mesenchymal cells are evidently somewhat sticky, for one finds frequently that two exogastrulæ fuse, telosynaptically as it were, by the ends of their guts (not laterally as in Herbst's cases). These adhere closely and more or less permanently to form "*fused twins*" (Fig. 3, *w*, *x*) whose parts, of varying relative size, have an interesting history of inhibition and reciprocal overgrowth, separation, etc., that might be followed further with profit.

Meanwhile differentiation has progressed somewhat in the *ectodermal* component also; especially if this is fairly large, has not been too strongly inhibited, and is then allowed to recover partially in sea water. Notable deviations from the normal development are seen in the marked reduction and generally radial disposition of apical structures. The mouth usually fails to form, but when it does it is practically terminal. The ciliary bands, when formed, appear as an apical ciliated tuft, plate, or small transverse ring. Apical outgrowths are common (Fig. 3, *d*). By the third day skeletal spicules have often formed in the mesenchyme, which is collected, not in two lateral masses near the blastopore, but, rather, crowded far forward into the apical end of the cœlome; these spicules stimulate the production of the pluteus arms, which are uncommonly close together and narrow angled from the marked reduction of the oral lobe and also from the tendency of the "anal" arms to be formed very near the oral region. Herbst also noted ('95, *a*, Figs. 32–36) that lithium larvæ successfully recovering in sea water continue their overproduction of mesenchyme and skeletal structures and

lay down an excess number of skeletal spicules (3, 4, or 5—the total final number in ordinary development—or even 7, in *Sphærechinus*) and the pluteus arms are increased in number and displaced accordingly and had besides an increased number of radii (4 or 5 instead of 3).

N/200 LiCl slows down development noticeably, but the blastulæ are of nearly normal size and less crowded with mesenchyme, which is extruded from the posterior end in only about half of the embryos. When gastrulation occurs there are found about 60–70 per cent. entogastrulæ and only 30–40 per cent. exogastrulæ and intermediate forms. The blastulæ recovering in sea water quickly resume active movements and become gastrulæ differing from the lithium larvæ in the slightly thinner ectoderm and entoderm; in the incomplete or merely temporary (Fig. 3 *g*, *j-m*, *p*) evagination of the gut; in the relatively large

FIG. 5 Sand-dollar embryos as modified by $CuSO_4$, $HgCl_2$, KNC, and dilute sea-water. *a*, an early exogastrula after 1 day in $CuSO_4$, M/1,250,000; *b*, after 2 days from $HgCl_2$, 3M/10,000,000; *c–e*, from diluted (80 per cent.) sea-water; *f-m*, from KNC M 750,000.

gastrular wall and more fully divided gut; and in their larger oral region and occasional belated evagination of the stomadeum.

N/250 LiCl yields mostly ordinary forms, but little retarded, and only 5–10 per cent. exogastrulæ. In still lower concentrations development quickly approaches that of the controls.

*Other agents*, such as KNC, $CuSO_4$, $HgCl_2$, HCl, $CaCl_2$, NaCl, and conditions, such as diluted or staling water, used in appropriate concentrations and intensities, were found to cause the same sort of general inhibition of development as LiCl, stopping entogenetic changes at cleavage, blastula, or gastrula stages; all may be employed to show the relatively greater inhibitory effect on apical and ectodermal parts; many brought the blastula up to a stage (Fig. 5) precisely simulating late Lithium blastulæ; and a few produced a small percentage of imperfect exogastrulæ, as in Fig. 5.

*Arbacia punctulata and Strongylocentrotus franciscana.*—In these sea-urchins a range of types of abnormality was produced

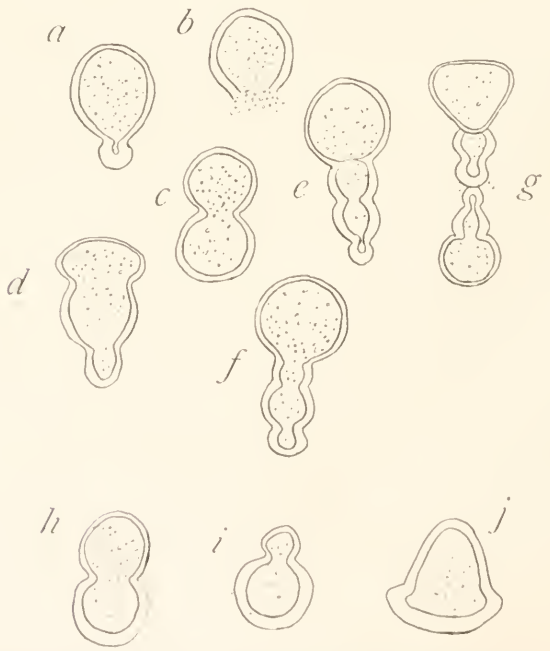


FIG. 6. Exogastrulæ, Constriction Forms, and Double Embryo of *Arbacia*, produced by LiCl and NaCl. *a–g* from LiCl, M/80 to M/100; *h j*, from NaCl, M/4 to M/8.

by lithium treatment, deviating from the normal in the same ways and essentially to the same degrees as do the sand-dollar lithium larvæ. The concentrations required are rather higher, the most numerous and typical lithium effects being induced in the Atlantic species by about N 80 to N 100 LiCl, and in the Pacific form by N 100 or less.

Several hundred sketches of teratological types in these urchins (the very common types seen in Figs. 6, 7) conform so

FIG. 7. Modified *Strongylocentrotus* Embryos. *a–e*, from LiCl, M 200; *f–l*, from LiCl, M 150; *m–o*, from NaCl, M 110; *p–q*, from KNO$_3$ M 250 000; *r–z*, from CaCl$_2$, M 160 to M 200.

closely to the sand dollar forms just described and to the modifications illustrated and described in such great detail by Herbst ('92, '95*a*, '95*b*) for *Echinus microtuberculatus*, *Sphærechinus*

*granularis* and *Strongylocentrotus lividus*, that further description would hardly aid in our present purpose of analyzing and interpreting the lithium effect.

It is important to mention the production of practically typical lithium embryos without the aid of lithium. Herbst found a good substutute for the lithium salts in Na butyrate etc., and Driesch ('95) obtained a high percentage of exogastrulæ from development at 30° C. for nearly a day preceding gastrulation. So it is not entirely surprising to find that in $CaCl_2$, N/80 to N/160, often the majority of *Strongylocentrotus* eggs become characteristic exogastrulæ, etc. (and many of these fused twins) practically indistinguishable in type from those in Figs. 1–3. Quite similar exogastrulæ are produced in *Arbacia* by NaCl, N/4 to N/8, and in *Strongylocentrotus* by N/8 to N/20 NaCl.

*Asterias forbesii and Orthasterias sp.*—Starfishes are by no means as responsive as the urchins and sand-dollar to the lithium action. Though all characteristic stages of general inhibition are

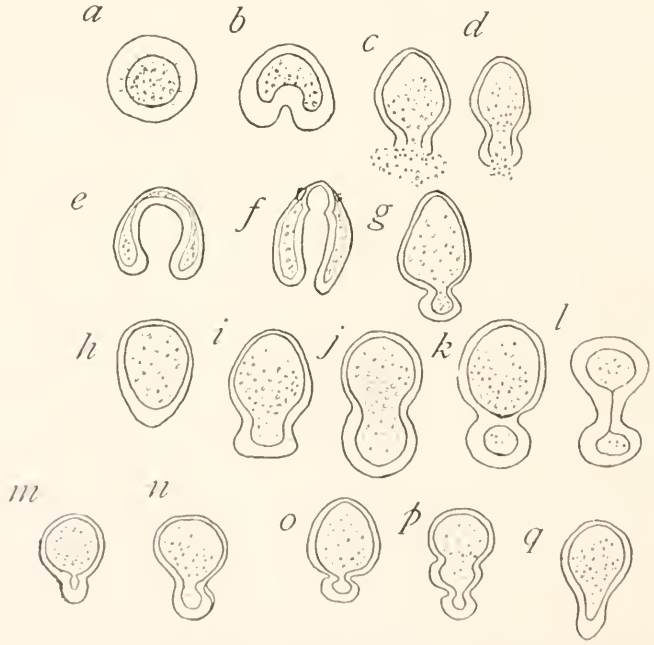


FIG. 8. Experimental Alterations of *Asterias*. *a–e* induced by LiCl, M/100 to M/160; *m–p*, induced by 75 per cent. sea-water; and *q*, by KNC, M/25,000

readily obtained by the lithium salts and other agents, and apical inhibition is evident in the characteristic reduction or entire suppression of the preoral ciliary field, etc., yet the endogastrulation process is not so easily interfered with.

But partial and occasionally fairly typical exogastrulæ occur with great regularity in *Asterias* cultures in N/100 to N/200 LiCl solutions in KNC, in diluted sea water, etc. (Fig. 8).

In *Orthasterias* N/160 LiCl sometimes induces exogastrulation or marked change of proportions of the germ layers, and not uncommonly in later development the stomadeal pouch is turned outward and forward from the mouth by a process apparently similar to exogastrulation (Fig. 9).

FIG. 9. Alterations of *Orthasterias* embryos in M/160 LiCl. *d–e*, showing stomadeal eversion; *c*, with mixed endo- and exo-gastrulation; *a–b*, with reduced ectoderm and greatly enlarged endoderm, particularly in the fore-gut. Note the position of the ciliary bands in *c–e* and in FIG. 8.

*Comparison of Susceptibility gradients of Normal and Lithium Embryos.*

1. *The Normal Embryos.*—The susceptibility relations in the early ontogeny of *Asterias* and *Arbacia* have been described by Child ('15*b*; '16*a*); these observations have been confirmed and extended to include the sand-dollar (data by Dr. Child and the writer) and the Pacific coast forms.

According to the direct susceptibility method (p. 61) the fertilized or dividing eggs, blastulæ, gastrulæ and larvæ were placed in lethal solutions of KNC (M/100 to M/1000), $HgCl_2$ (M/50000 or less), $CuSO_4$, concentrated neutral red or methylene blue, and to a few other salts, etc. and the course and relative times of death of parts noted by the criteria of possibility of recovery in return to sea water and of disorganization of cell structure. Disorganization is often made more visible either by a brief return to sea water, or by previous or simultaneous staining in

dilute neutral red since the stained tissue decolorizes sharply at the death point. Since general susceptibility increases steadily from fertilization to gastrulation the concentration of the agents was correspondingly decreased in the later stages.

The order of susceptibility in the succeeding stages of development is shown in Fig. 10. Disorganization, manifested by droplet

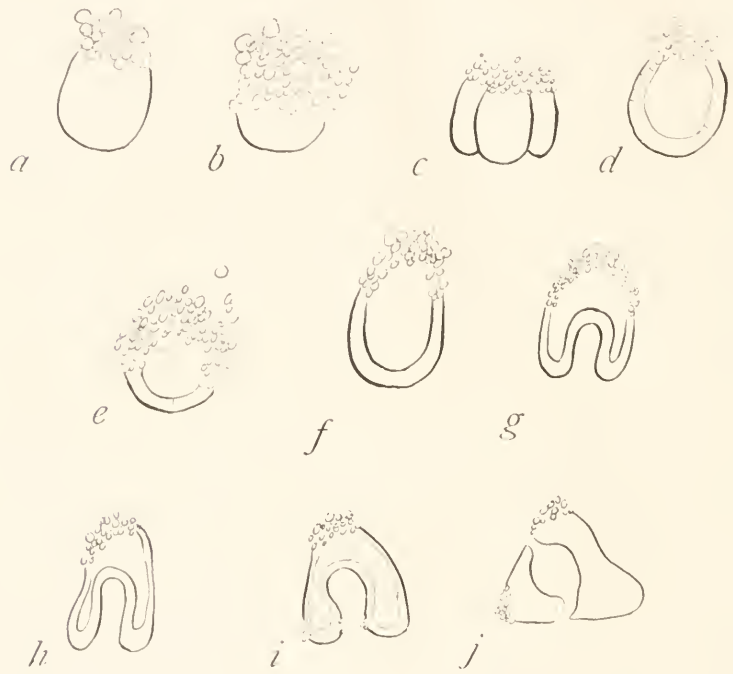


FIG. 10. Disintegration of normal sand-dollar eggs and embryos, showing relative susceptibility of parts. Mesenchyme omitted. *a–b*, eggs; *c*, 4-celled; *d f*, blastulæ; *g–j*, gastrulæ and early larvæ. When the polar axis becomes recognizable disintegration proceeds from animal to vegetal pole (*i.e.* is *apico-basal*).

formation, cell separation, roughening of contour, visible disintegration, and decolorization, according to the particular agent used, begins at one point of the surface of the egg, or at one pole of the blastomeres in the 2- or 4-cell stage, or at one pole of the cleavage mass or early blastula, and then extends gradually over adjacent parts to reach last the opposite surface or pole.

As soon as the blastula differentiates to show the basal thickening and begins its elongation, one may orient the embryo in

its chief apico-basal axis at a glance. Here the manifestations of death are first visible at the animal pole or apex and progress gradually and steadily towards the basal region which is reached last, often hours after the opposite end had been affected. The apparent reverse in some slow-acting solutions was obviously due entirely to the basal extrusion of the mesenchyme.

The same gradient persists into the gastrula stage, where the apex shows the greatest susceptibility, while the invaginated entoderm is so much more resistant (except probably for a zone around the blastopore) that it usually survives in M/100 KNC an hour or more after the ectoderm of the entire gastrular wall has disintegrated. By returning non-disintegrating gastrulæ to sea water after an appropriate exposure the ensuing disintegration may be checked midway in its course and recovery larvæ are obtained showing absence or partial loss of apical (or even more basal) ectoderm and more or less complete survival of the entodermal parts which are accordingly large in proportion to the whole. As in the case of *Arbacia* "in the gastrula the apex of the conical body represents the apical end of the major axis, and the blastopore the basal end, while the entoderm represents a still more basal region of the egg and blastula, and the mesenchyme the most basal region" (Child, '10*b*, pp. 68–69).

Vital staining with neutral red and other basic dyes shows an apico-basal progression in blastulæ and early gastrulæ quite paralleling in all essentials the wave of advancing disintegration as described above.

The experimental embryologists have described the common

FIG. 11. Diagram illustrating the polar arrangement of parts in the egg (*a*) in the normal embryo (*b*), and in lithium embryos (*c*) of echinoderms. 3, 2, 1, fore-, mid-, and hind-gut. The lithium embryo is really an elongated and differentiated egg, with the germ-layers localized in the same order as were their antecedents in the egg.

plan of organization of the Echinid egg as such that the prospective significance of different levels of the egg along its main structural axis is as follows (see Przibram, '08): The animal pole half (diagram, Fig. 11. Ect.) furnishes the ectoderm and its differentiations; the subequatorial zone (End.) the gut and its derivatives; and the vegetal pole (Mes.) the primary mesenchyme and the larval skeleton. In Fig. 11 (*b*) and (*c*) these parts are homologized in the normal and the lithium-modified gastrulæ

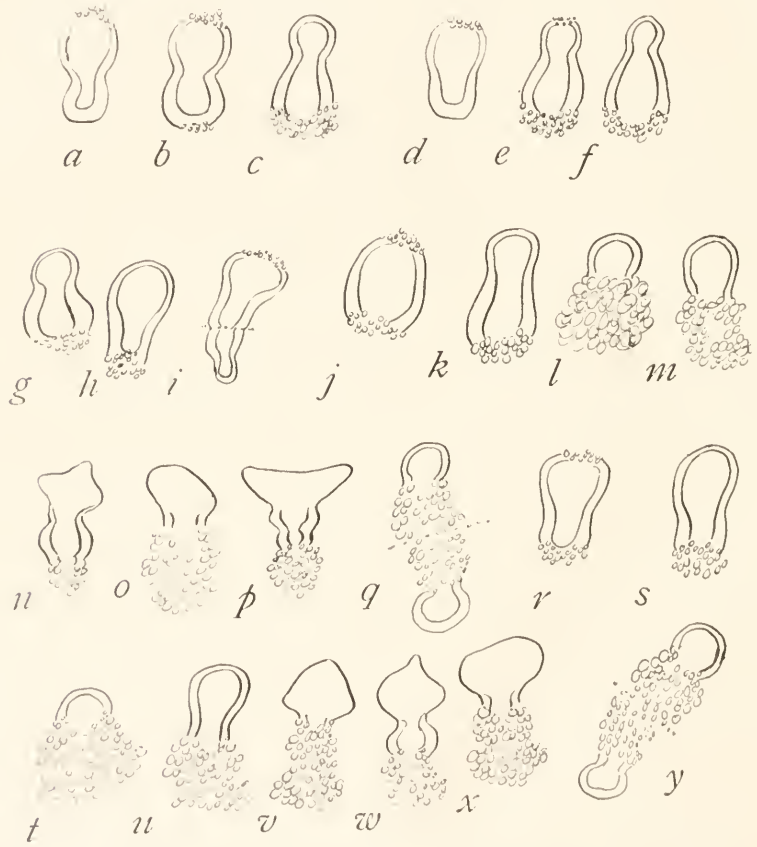


FIG. 12. Disintegration of typical lithium-modified sand-dollar embryos and larvæ. Mesenchyme omitted. *a–c* in neutral red; *d–i*, in $HgCl_2$ M/500000; *j–q*, in $HgCl_2$ plus Neutral Red; *r–y*, in KNC, M/1000; *i*, *q*, *y*, fused doubles. In the less modified forms (*a*, *b*, *d*, *j*, *r*) susceptibility is about equal apically and basally. In the more modified forms with large development of endoderm a distinct *basi-apical* disintegration gradient is always found. In general the larger the relative size of the endoderm and of the fore-gut the more distinct is this reversed gradient.

respectively. Obviously in the lithium-modified embryo the egg has merely elongated and differentiated without the usual infolding.

2. *Lithium Embryos.*—Tested with the same agents in precisely the same way as were the normal embryos the exogastrular and constriction forms (p. 63) showed new and different and characteristic susceptibility relations. General susceptibility is much less than in the normal, and the original apico-basal gradients of susceptibility, vital staining and decolorization are less marked, absent or reversed (Fig. 12).

Quite commonly the basal end of the entoderm has already begun disintegration in the LiCl solution itself, and much of the secondary mesenchyme may have been removed and the primary mesenchyme may have escaped from the blastocœl (Fig 12, *l*, *l*).

Newly formed lithium exogastrulæ and constricted forms removed intact to M/1000 KNC, M/500000 $HgCl_2$, or concentrated neutral red, disintegrate in the next few hours. In this process disintegration and decolorization after staining generally begins in the secondary mesenchyme-forming region of the archenteron.

Sometimes the two extreme ends disorganize at the same time, the apical ectoderm of the gastrula wall being as susceptibile as the basal entoderm of the archenteron. And in a few cases the ectoderm goes a little in advance of the opposite end. In these hour glass forms the susceptibility varies with the size of the components; the larger component being quite uniformly the more susceptible; and indeed it is apparently susceptible in direct proportion to its relative size, as the sketches suggest. The greater the reduction of the ectoderm the higher is its resistance.

Exogastrulæ removed from the lithium in a more differentiated state with bi- or tri-partite gut, etc., exhibit regularly the reversed order of susceptibility of parts (Fig. 12).

## Discussion and Interpretation.

### 1. *Physiological Gradients in Echinid Embryos.*

It will be taken as highly probable that in the egg and blastula of the echinoderms so far studied the susceptibility declines from the apical animal pole region to the basal vegetal pole levels. In

the late blastula, gastrula and larva the susceptibility demonstrably declines sharply from ectodermal to entodermal and mesenchymal regions (p. 74). This is chiefly significant as indicating a corresponding gradient of metabolic activity—as a prerequisite to normal development—which is further evidenced and manifested by structural differences along the apico-basal axis (differences in cell size, cilia, pigment, vacuolization of protoplasm, etc.) and by functional differences in rate of developmental processes (growth, cell division, morphogenesis, and differentiation).

It is a familiar fact that teratogenic agents and conditions applied to whole eggs and embryos affect different regions of the developing organism differently and to a different degree. On the quantitative metabolic gradient conception regions of greatest susceptibility are subject to the greatest retardation in the developmental processes, while those of lowest susceptibility are correspondingly least inhibited, as is experimentally proven for *Arbacia* (Child, '16*a*), Annelids (Child, '17), the frog (Bellamy, '19), etc. Thus most teratological forms may be reproduced according to expectation and explained consistently.

Lithium effects also seem to be produced by a differential inhibition along a quantitative metabolic gradient. The facts relevant to this interpretation have been collected in the next section.

### 2. *Analysis of the Lithium Effects.*

Exogastrulation aside, lithium larvæ are characterized *morphologically* by the abnormal proportions of their parts, which involve all the germ layers in a manner we may summarize as follows:

(*a*) *Increase in amount and change in position of the mesenchyme and its derivatives:*

Excess mesenchyme of stereoblastulæ (p. 63).

Excess pigment cells (p. 63).

Excess skeletal structures, *e.g.*, spicules, arms, radii of arms, etc. (Herbst).

Conspicuous production of serous fluids and swelling of serous cavities in many annelid, fish, and amphibian embryos.

(*b*) *Hypertrophy of entodermal parts, especially basally:*

Relative and absolute increase of size of the gut in general, and of the fore-gut in particular (Figs. 2, 3, 4 etc.).

Thickness of entodermal wall (p. 68).

Richness of entoderm in nuclei (p. 68).

Small size of entodermal cells (Herbst; for frog, Bellamy '19).

(*c*) *Relative and Absolute Diminution of the Ectoderm, especially apically:*

Reduction of the ectodermal component of the hour glass forms (Figs. 2, 3).

Retardation of the apical region in differentiation (p. 69).

Retention of radial symmetry (p. 69).

Sparseness in nuclei (Herbst).

Large size of ectodermal cells (Herbst, Bellamy, Le Plat, '20, et al.).

Failure of ectoderm to grow down over the entoderm in the frog (Gurwitsch, '04, Morgan, '03*b*, Bellamy, '19, Le Plat, '20).

Since the main object of this paper is to deal with the lithium effect *physiologically*, it will profit to restate the morphological data just summarized in physiological terms:

Sufficiently strong concentrations of the Li ion will suppress development at any early or late stage of cleavage, of the blastula, etc. Slightly lower concentrations also retard development, but this retarding influence is most marked in the most susceptible region (ectoderm) of the embryo, distinctly less marked in the less susceptible entoderm, and least marked in the least susceptible mesenchyme. The last tissue undergoes a relatively rapid and excessive growth and abundant proliferation (in many ways resembling malignant tumor growth) to fill the blastocœle and lay down the anlage of an increased number of skeletal spicules (which at once push out the arms of the pluteus if recovery is permitted). The change of position of the mesenchyme may indicate a tendency (active or passive) to occupy spaces near cells of lowest metabolic activity.

The entoderm growth zone extends itself apically on to the gastrula wall (more and more as the gradient is levelled down or reversed) by virtue of an enhanced mitotic and growth activity, which is one of the most distinctive characters of a lithium embryo

and the feature by which the lithium effect may best be quantitatively estimated. The greater the lithium effect short of general inhibition, the greater the relative size of the entodermal as compared with ectodermal parts.

Meanwhile mitosis and growth have slowed down or utterly ceased in the ectodermal zone, whose development varies inversely with the development of the entoderm. In extreme cases the entodermal growth zone overspreads the whole blastula wall resorbing and incorporating and changing entirely the prospective significance of the apical region. This resorption doubtless proceeds in proportion to the relative rate of metabolic activity of the entoderm; just as in normal ontogeny the ectoderm feeds upon and limits the development of the gut, or as the nervous system of a starving animal maintains itself at the expense of parts of lower physiological activity. Here the ectoderm, usually the master germ layer, loses control and the more active entoderm gains the ascendency and may become the major part of the embryo. That the fore-gut of the evaginated archenteron also increases disproportionately may be attributed to the same process.

The inhibitory effect of lithium appears in the order: ectoderm > entoderm > mesenchyme; in the ectoderm itself: apical > basal; and in the entoderm: hind-gut > mid-gut > fore-gut (Fig. 13).

The most substantial direct proof of this reversal of the normal physiological gradient is found in the more or less complete reversal of the usual order of susceptibility; for in extreme cases the direct susceptibility was seen to be greatest in the fore-gut region and to decline towards the hind-gut and ectoderm (Fig. 13).

Judged then by the criteria of susceptibility, of developmental activity in mitosis, growth, rate of differentiation, etc., the metabolic gradient of the lithium larva is diminished, obliterated, or in the extreme case actually reversed (basi-apical). It is evident, however, that the reversal of the gradient occurs too slowly or too late to alter significantly the nature of the differentiated end products, for the respective germ layers bear recognizable similarity to these of the normal larva. Earlier exposure of the egg and a different technique of recognizing polarity would be neces-

sary to determine whether lithium can be made so far effective as to cause ectoderm to appear at the basal pole, etc.

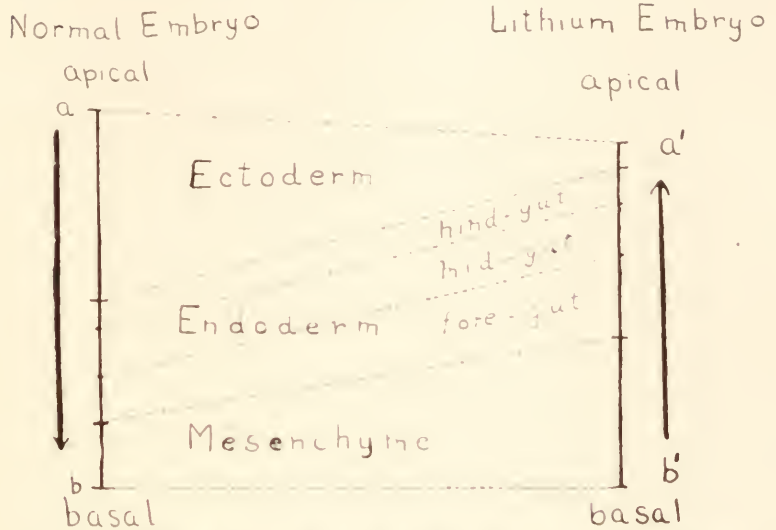


FIG. 13. Diagram showing the changed proportions of parts in a lithium embryo (right) as compared with a normal embryo (left), and the typical reversal of susceptibility (the arrows), $a$–$b$ in normal, $b'$–$a'$ in lithium embryos. The effect of a depressing concentration of lithium is to shift the embryo to the right. An accelerating agent would alter embryonic proportions in the opposite direction.

### 3. *Non-specificity of Lithium Effects.*

(*a*) *Changes of Proportions of Parts.*—A great variety of chemical agents and physical conditions have been employed in experimental embryology to cause general death and disintegration, general inhibition of development, differential (*i.e.*, localized) inhibition, and, in the minimal effective concentrations, differential acclimation effects. The striking fundamental similarity of teratological forms produced by diverse agents and conditions is too remarkable to be insignificant. There can be no one teratogenic substance or condition whose effect differs sharply in kind from others. Though each may have its own mode of attack, the end result is in general injurious and inhibitory or accelerative, according to the power of resistance of different levels along the metabolic gradient. The only specificity of lithium effect seems to lie in the higher regional differential susceptibility of many organisms to it; lithium has a particularly

marked capacity of inhibiting locally, retarding apical regions without appreciably hindering basal regions.

(*b*) *Exogastrulation.* In practice quite typical exogastrulae are obtained in the sand dollar or in sea-urchins by all the lithium halides, sodium butyrate, KCl, etc. (Herbst), by $CaCl_2$, dilution or staling of sea water, etc. (this paper), by elevation of temperature to 30° C. during gastrulation (Driesch); and initial stages of exogastrulation by many other agents, as NaCl, $CuSO_4$, neutral red, etc. As regards exogastrulation specificity appears to reside chiefly in the biological material rather than in the external agent, for perhaps only in the pluteus-forming echinoderms is the lithium effect "typical"; in the starfishes, *Crepidula* (Conklin), and the sponge, *Oscarella* (Maas, '98) the effects are sufficiently marked and similar as to be recognizable; and in the annelids, ascaris, amphioxus, frogs (?) only negative results appear. Possibility of inducing exogastrulation depends, doubtless, on the specific constitution and organization of the egg, especially on the polar arrangement of the levels destined to form ectoderm, entoderm and mesenchyme. The physiological equivalents of exogastrulae may well appear quite different morphologically in different organisms.

### 4. *The Mode of Action of the Lithium.*

The problems of lithium action resolve into the causes of the two essential modifications: first, the disproportionate development of entoderm and mesenchyme; and second, exogastrulation. These are without doubt closely interrelated processes proceeding from a common fundamental cause.

Herbst admitted that he found himself in complete darkness on these matters but gave reasons for his opinion that the site of action of the salt must be in the egg itself rather than in its external membranes, and that $Li'$ must produce a specific stimulating effect on vegetal pole and entodermal cells, whose capacity for taking up and retaining this ion he believed to be greatest ('95*a*, p. 185). Seeking to put the matter on a physico-chemical basis one of his students (Spek, '18) gave some evidence that if $Li'$ did penetrate into certain (*e.g.*, entodermal) cells it would make for a greater imbibition of water by their colloids. Driesch ('95) believed that "the growth process of the blastula wall is

the vital fundamental process; the direction towards which the growth proceeds in exogastrulation is determined by the surroundings." It is suggestive that both these leading experimentalists appealed to some more or less differential growth process; but in neither case was the evidence more than meager, indirect, and isolated.

Attacked from the new viewpoint of differential inhibition, etc., lithium action is seen to be interpretable in a way not only entirely consistent and coördinate with a vast body of modern work in general and special physiology and of physiological form determination and teratomorphy, but also substantiated by considerable direct proof and many suggestive parallels (p. 78, and Child, '20). Since Li' in the concentrations actually necessary is certainly inhibitory and since the inhibitory effects are felt chiefly at the animal pole and in its differentiations and least in the mesenchyme and the entoderm, it is unlikely that the Li' acts so much directly on the basal regions; it may be said rather to *release* these regions from the control of the ectoderm by checking the (usually dominating) activity of the latter.

To determine the more proximate causes of the inhibitory effects will require much further detailed observation and experiment (for each separate agent) on sites and rates of penetration, water imbibition and withdrawal, alteration of surface tension and chemotaxis (Rhumbler, '02), precipitations, gelations, solutions, etc. Into whatever ultimate causal components resolved the end result remains, physiologically, a differential inhibition.

Exogastrulation is evidently a secondary manifestation of this differential action along a levelled or reversed metabolic gradient. Since the lithium-modified embryo is merely a lengthened and differentiated egg in which endogastrulation has been prevented (Fig. 11) exogastrulation might be considered even a direct result of the inhibition of these eggs (levelling the normal gradient necessary for endogastrulation).

But a mechanical factor seems to be involved. Expansion and overgrowth of the ectodermal epithelial layer is checked by the failure of the usual multiplication and enlargement of its cells, while growth and cell division of entoderm are less, and those of mesenchyme least retarded. Consequently the ectodermal cells

are few and large while the entodermal layer is rich in smaller nuclei. The mesenchyme cells proliferate in enormous excess and fill and overdistend the small blastocœl. Thus considerations of space and inner pressure alone render almost mechanically impossible the infolding of the entoderm which is, further, much longer, larger and thicker than usual, and is contiguous with the ectoderm by a vastly enlarged blastopore ring.

Certain other relevant facts should be noted: that the lumen of the exogastrular gut is really a part of the blastocœl or cœlome and is not the true gut cavity; that the constrictions of the differentiating gut are also therefore reversed in direction; that the stomadeum also often everts in starfish; that exo- and endogastrulation intergrade (there may be first one and then the other, or both at once). It is possible that the agent in obliterating and reversing the primary physiological gradient of the organism as a whole at the same time obliterates and reverses the functional polarity of the separate cells of the epithelial layers, so that insofar as polarity of cells aids in normal endogastrulation it might act in the opposite way in the lithium larvæ (by failure of the cells to widen and vacuolate basally, change their nuclear position, etc. cf. Gudernatsch, '13).

Tests of this interpretation of the lithium action may be made by an appropriate modification of the technique on other gradients (embryological, regenerating, motor). This agent might tend to reverse the polarity of regenerating pieces (*e.g.*, hydroid internodes, as an electric current does, Lund, '21), or of motor gradients (cilia, or muscular peristalsis), etc.

## Summary and Conclusions.

1. Typical lithium larvæ may be produced from a high percentage of eggs of the sand-dollar, *Echinarachnius parma*, and of two sea-urchins, *Arbacia punctulata*, and *Strongylocentrotus franciscana*, by addition of LiCl to the sea water. These types approximate closely those obtained by Herbst in other urchins.

2. Morphological features common to and characteristic of these larvæ are (p. 78): (*a*) exogastrulation in many cases, and (*b*) always increase of mesenchyme and of entoderm and a compensating decrease of ectoderm. Further the most apical parts of the ectoderm and its differentiations are most decreased, and

the most basal parts of the gut are most increased, as the lithium alteration becomes maximal.

3. In the lithium larvæ the order of direct susceptibility of parts to quickly killing agents is essentially the reverse of that of the normal gastrula and larva: the gradient of susceptibility in the normal egg and blastula is apico-basal, and declines from the ectoderm to entoderm and mesenchyme in the gastrula and larva, while in the fully formed exogastrula the susceptibility of the ectoderm (gastrula wall) is least, that of the entodermal parts more, and that of the mesenchyme probably greatest of all. A similar reversal of the usual susceptibility is met with in parts of the differentiated gut of the lithium larva.

4. "Lithium larvæ" are not the specific product of the $Li'$ ion alone, but are readily obtained in $CaCl_2$, diluted or stale sea water, by Na butyrate (Herbst), by heat (Driesch), and in less typical form by many other salts, basic dyes, etc.

5. The specificity resides rather in the biological material than in the agent, but $Li'$ appears to possess in high degree the power of localized action: *i.e.*, its inhibition is markedly regional, slowing down or stopping development of the most apical parts, while hindering little or inappreciably development of the basal parts.

6. These induced, highly differential, rates of growth and differentiation lead directly or indirectly to exogastrulation in extreme cases; perhaps largely because of the mechanical impossibility of invaginating a greatly enlarged entoderm into an unusually small blastocœl, itself overcrowded by a precocious and excess proliferation of mesenchyme.

7. These changes in proportions and positions of parts in "lithium embryos" appear to follow as naturally and necessarily from the altered metabolic relations (more or less complete reversal of the physiological gradients), as the normal proportions and positions of parts follow from the normal metabolic relations.

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# THE EXISTENCE OF A SHORT REPRODUCTIVE CYCLE IN *ANODONTA IMBECILLIS*.

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Fresh water mussels of North America have been classed by Sterki (1895) as summer or winter breeders. He notes that in winter breeders fertilization of ova occurs in the late summer, embryonic development is completed and the fully developed glochidia are carried through the winter in the maternal marsupia and discharged in the following spring. In the summer breeders fertilization occurs in the late spring or early summer and the fully formed glochidia are discharged "as a rule by the end of August."

Lefevre and Curtis (1912) prefer to designate these two classes as long and short term breeders. They include *Anodonta* in the former group, and although *A. imbecillis* is not listed in the data from which their conclusions are drawn, the statement is made that the breeding season is a generic character, "all species of a genus having essentially the same period of gravidity."

In a table compiled by Coker, Shira, Clark, and Howard (1919) gravid *Anodonta imbecillis* are listed for all months with the exception of April.

*Anodonta imbecillis*, although not important commercially, is of interest for two reasons; first, it is one of the two known species which carry the young in the marsupia during metamorphosis from glochidia to juvenile mussels, thus eliminating the parasitic stage on fishes which other species undergo, and, second, it is one of the few species which are hermaphroditic (Sterki, 1898).

During the summer of 1922 the writer attempted to work out a method of aparasitic mussel culture of commercially valuable mussels. One line of attacking this problem which seemed to offer promise of results was to simulate conditions pertaining in metamorphosis during the intra-marsupial life of aparasitic forms. Therefore a series of observations was begun on *Anodonta imbecillis* during the course of which successive examinations

were made at intervals of several days on more than one hundred individuals. This has furnished the following evidence for a shorter reproductive cycle than formerly reported for this species.

## Material and Method.

*Anodonta imbecillis* is commonly called the "paper shell" because of the extreme thinness of the valves. Possibly correlated with this economy in shell formation, is its rapid rate of growth. It may begin to reproduce during the second year. The observations in this paper were made on two- and three-year-old mussels.

In this species the outer gills serve as marsupia. It is an easy matter to insert a capillary pipette between the valves, make a single puncture in the marsupium, and remove some of its contents with only slight injury to the mussel. This puncture heals rapidly. In order that the least possible modification of reproductive activity might be produced by this examination, the samples removed were small, containing only from 20 to 40 individuals. The puncture was made in a direction parallel to the long axis of the gill so that the sample removed contained young from several gill compartments.

Relatively slight disturbances may cause "abortion" of marsupial contents in some species. Lefevre and Curtis, however, note that they have never observed abortion in *Anodonta*. The thin shell of *A. imbecillis* renders the above described operation very simple as the valves may be opened for the insertion of the pipette with the thumb nails. This probably eliminates the possibility of abortion as a factor bearing upon the following evidence.

The examinations extended over a period of one and one half months from the middle of July to early in September.

Most of the mussels studied were taken from the main supply reservoir of the U. S. Fisheries Biological Station at Fairport, Iowa, which is supplied daily from the Mississippi River. A few preliminary observations were made on mussels taken from the river.

Throughout the observations they were kept in running water in experimental troughs supplied from a pond containing an abundance of pond life. As opportunity for considerable sedi-

mentation was afforded, this supply was much clearer than water from the river or reservoir. The fact that very successful yields were obtained in artificial mussel culture experiments carried on simultaneously under these conditions by Dr. A. D. Howard and B. J. Anson excludes any possible intervention of adverse environmental conditions.

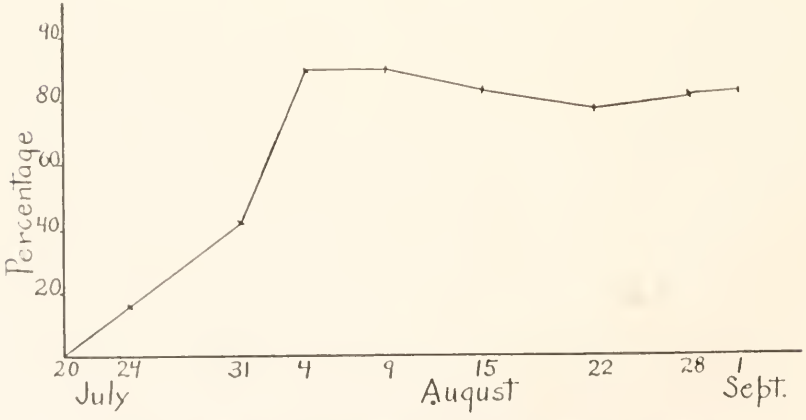
## Observations.

Between July 15 and 20 about two dozen mussels were examined without finding a gravid individual. On July 24, 5 of 33 examined (15 per cent.) contained early embryonic stages. Nineteen of these 33 were observed repeatedly until September 1. On July 31, 8, including the five already noted (42 per cent.), contained embryos. On August 4, 17 or 89.5 per cent. were gravid. Larger numbers of this species were examined during August. From August 8 to September 1 the percentage of mussels bearing embryos, glochidia, or young in various stages of metamorphosis never dropped below 77.2 per cent. (see Table I. and Graph 1).

TABLE I.

Percentage of Mussels Bearing Embryos or Glochidia.

| Date | 7/15–20 | 7/24 | 8/4 | 8/9 | 8/15 | 8/22 | 8/28 | 9/1 |
|---|---|---|---|---|---|---|---|---|
| Number examined | 20–24 | 33 | 19 | 69 | 101 | 105 | 103 | 57 |
| Percentage | 0 | 15.1 | 89.5 | 89.9 | 83.2 | 77.2 | 80.6 | 82.5 |




Graph 1. Percentage of mussels bearing embryos or glochidia.

The conclusion drawn is that the reproductive season of *Anodonta imbecillis* in this location begins during the latter part of July.

As these observations were continued the various stages of embryonic development and glochidial metamorphosis were identified and data accumulated as to the time required for this transition. Eleven different stages were identified as follows:[1]

1. Early segmentation of the fertilized ovum.

2. Later segmentation.

3. Early differentiation of the single anlage of the valves which at this stage caps the segmenting cell mass.

4. Formation of a groove in this "cap" which divides it into two separate valve anlagen.

5. The developing valves lie on the segmenting cell mass "like continents in relief on the globe."

6. The two valves resemble a folded leaf which now completely encloses the future "soft parts" of the young larva.

7. The glochidial shape is attained but the larva is still colorless and transparent. The single glochidial adductor muscle can be seen to quiver or "flash" although the valves are closed.

8. The glochidia are now fully formed, have a rich yellow color, and a single adductor muscle. Many may be seen to snap vigorously if freed from the egg membranes in the process of removal from the marsupia. Those within the membranes have their valves opened. This stage is equivalent to that at which the glochidia of species dependent on parasitism on fishes for metamorphosis are extruded from the marsupia.

9. Mid-metamorphosis; rich yellow in color, no adductor muscle visible.

10. Late metamorphosis; the two definitive adductor muscles are now visible. Snapping of the valves cannot be induced by freeing the glochidia from their egg membranes.

11. Juvenile mussels; the foot is now visible, active snapping of the valves and extension of the foot occurs.

[1] No attempt was made to describe fully the various stages of development; they are merely characterized for greater accuracy in determining the duration of developmental stages. Examination was made with a binocular microscope magnifying × 50.

Even juvenile mussels are found within the egg membranes. A stringy transparent gel forms or is secreted about the egg membrane enclosing the developing embryos after they reach the marsupia. It is well formed by the stage at which they begin to turn yellow and partly disappears in late metamorphosis.

Table II. includes observations on twenty typical individuals. More than one hundred are included in the data from which the average time required for the transition between stages is determined.

TABLE II.

STAGES OF DEVELOPMENT OF YOUNG IN MARSUPIA OF *A. imbecillis*.[2]

| No. of Mussel. | Date. | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 7/24 | 7/27 | 7/31 | 8/4 | 8/9 | 8/15 | 8/22 | 8/28 | 9/1 |
| 1 | 3 | 4 | 6 | 7 | 8 | 9 | 0 | 0 | 1 |
| 2 | 3 | 4 | 6 | 7 | 8 | 9 | 0 | 3 | 4 |
| 3 | 3 | 4 | 5 | 6–7 | 8 | 9 | 0 | 1 | 1–2 |
| 4 | 6 | 7 | 8 | 9 | 0 | 0 | 0 | 0 | 1 |
| 5 | : | : | 3 | 4 | 6 | : | 9 | 0 | 1 |
| 6 | : | : | 3 | 4 | 6 | 8 | 9 | 0 | 0 |
| 7 | : | : | 7 | 8 | 9 | 0 | 0 | 1 | 3 |
| 8 | : | : | : | 8 | 9 | 1 | 3 | 7–8 | 9 |
| 9 | : | : | : | 8–9 | 0 | 0 | 1 | 4 | 7 |
| 10 | : | : | : | 5 | 7 | 8–9 | 0 | 0 | 3 |
| 11 | : | : | : | 6 | 7 | 8–9 | 0 | 1 | 3 |
| 12 | : | : | : | 5 | 7 | 8–9 | 0 | 0 | 3 |
| 13 | : | : | : | 1–2 | 5 | 7 | 8–9 | 0 | 3 |
| 14 | : | : | : | 1 | 5 | 7 | 9 | 1 | 4 |
| 15 | : | : | : | 3 | 4 | 6 | 8–9 | 0 | 3 |
| 16 | : | : | : | 4 | 6–7 | 7–8 | 9 | 0 | 1 |
| 17 | : | : | : | 1 | 4 | 6 | 9 | 10–11 | 0 |
| 18 | : | : | : | 0 | 0 | 1 | 5 | 8 | 9 |
| 19 | : | : | : | 0 | 1 | 5 | 7–8 | 10–11 | 0 |
| 20 | : | : | : | 6 | 8 | 10 | 0 | 1 | 3 |

Numbers 13, 14, 17, 19 underwent a complete reproductive cycle in from 22 to 27 days during August. The calculation of cycle duration by averaging parts of cycles from all available data gives an average cycle duration of 3 to 4 weeks. This period then provides time for fertilization, segmentation, embryonic development to the glochidial or larval stage, and metamorphosis of the glochidium into the juvenile mussel. In *Anodonta imbecillis* it all occurs in the maternal marsupium, with the possible exception of fertilization. Metamorphosis, which in

[2] 0 in the table indicates empty marsupia during the interval between reproductive cycles. Numbers refer to stages characterized in the text.

other forms occurs in epithelial "cysts" on the gills of fishes, requires in this species only 7–10 days, as compared with a minimum of 12–14 in *Lampsilis anodontoides* and *L. luteola*. Development from the fertilized ovum to the fully formed glochidium may occur in two weeks' time.

The duration of the interval between reproductive cycles varies from 2 or 3 days to 2 (or rarely 3 )weeks.

It is realized that more extensive observations would provide material for more accurate determination of the duration of various stages of this cycle and the possible changes in these time relations during the fall and winter months.

However, the data at hand does warrant the conclusion that *Anodonta imbecillis* cannot properly be classed as a long term breeder. Juveniles are discharged during the latter half of August and first half of September and a new reproductive cycle begins. Whether this cycle is repeated throughout the months when this species is listed as gravid is still to be determined.

## Discussion.

The possible correlation of the rapid rate of growth and the early attainment of sexual maturity of *Anodonta imbecillis* with the thin "paper shell" has already been mentioned. It is possible that this short reproductive cycle may be correlated with these factors.

No attempt was made to determine whether self or cross fertilization is the normal process in *Anodonta imbecillis*. If self fertilization is the rule, hermaphroditism may have been a contributing factor in the establishment of a shorter reproductive cycle.

Since to date only two species have been reported as developing aparasitically, the idea that they may have depended at one time upon parasitic metamorphosis, but later evolved an ability to develop independently, seems natural. Many investigators have commented on the existence of small hooks at the valve edges as constituting the remains of a former parasitic adaptation. Since parasitism on fishes which make seasonal upstream migrations furnishes an efficient method of distribution (possibly the only method of extensive upstream migration of mussels), a wide distribution of aparasitic forms would lend added weight to

this theory. But if the natural course of evolution of reproduction be followed, parasitism must be considered a secondary development. It is possible therefore that metamorphosis without parasitism is the primitive method and that *Anodonta imbecillis* never has relied upon parasitism for development. It is also possible that the shorter reproductive cycle is the primitive one rather than an adaptation from one similar to that of other species of Unionidae.

## Summary.

*Anodonta imbecillis* cannot be classed with either long or short term breeders as defined by Lefevre and Curtis. In specimens obtained at Fairport, Iowa, a complete reproductive cycle (including metamorphosis from glochidia to juvenile mussels, for reproduction in this species does not depend on parasitism) is completed in from 3 to 4 weeks during the late summer. Another cycle begins after an interval varying from 2 to 3 days to 2 weeks. Gravid *A. imbecillis* have been found in all months but April. It is possible therefore that this short reproductive cycle is repeated throughout the year.

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# RESISTANCE OF RHABDITIS TO ACIDS.

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As is well known, *Anguillula aceti* lives in vineger and is highly resistant to acetic acid. Henneberg (1) has shown that this nematode can live in 13.5 per cent. acetic acid. Abbott and Richards have reported that it can live for more than 24 hours in Tellyesnicky's fluid, and for three or four hours in Gilson's fluid.

The writer intended to see in the first place whether other free-living nematodes, which do not come in contact with acids in their natural life, were resistant particularly to acetic acid as well as other acids. For this purpose *Rhabditis elegans* was chosen. In the course of the work it was found that *Rhabditis* is more resistant than the tadpole, *Daphnia*, *Aelosoma*, and *Paramecium* not only to various kinds of acids, but to other toxic substances as well. The cuticle with which nematodes are covered is known to be composed of a very resistant substance; experiments, therefore, were tried to determine whether or not the cuticle is responsible for this resistance, the worms, injured and uninjured, being stained in solutions of neutral red and of methylene blue. This work was begun at the suggestion of Dr. M. H. Jacobs, whom the writer wishes to thank for valuable advice.

Adult hermaphrodites of *Rhabditis elegans* and tadpoles about 10 mm. long and with external gills were used. *R. elegans* is found in decayed matter and is easily cultivated in a peptone solution. Every experiment was repeated at least twice, and 14 to 30 individuals were used for each experiment. The temperature at which experiments were done varied from 21° C. to 26° C.

As shown in Table I., *Rhabditis* is the most resistant to various kinds of acids. It might be noted that it can live about two hours in N/30 acetic acid, while *Daphnia*, the form next in order of resistance, can live only about an hour and a quarter in N/100 acetic acid. From the results of the experiments given in the table we notice, however, that *Rhabditis* is not particularly resistant to acetic acid as compared with other acids.

TABLE I.

AVERAGE TIME IN MINUTES REQUIRED TO CAUSE CESSATION OF ALL MOVEMENTS.

| | *Rhabditis.* | *Paramecium.* | *Aelosoma.* | *Daphnia.* | *Tadpole.* |
|---|---|---|---|---|---|
| HCl, N/100 | 60 | <1 | <1 | 23 | 12 |
| HCl, N/50 | 17 | <1 | <1 | 9 | 7 |
| Acetic acid, N/10 | 23 | <1 | <1 | 6 | — |
| Butyric acid, N/10 | 13 | <1 | <1 | — | <1 |
| Salicylic acid, N/100 | 26 | <1 | <1 | 13 | — |

Is this resistance of *Rhabditis* due to a mechanical reason or to the ability of the animal to neutralize acids? The results of the experiments with toxic substances (Table II.) seem to show that the first is the case, since *Rhabditis* is the most resistant of the five forms studied not only to acids, but to the other toxic substances as well. If this presumption is correct, the cuticle with which *Rhabditis* is covered may be supposed to prevent the penetration of the toxic substances.

TABLE II.

AVERAGE TIME IN MINUTES REQUIRED TO CAUSE CESSATION OF ALL MOVEMENTS.

| | *Rhabditis.* | *Paramecium.* | *Aelosoma.* | *Daphnia.* | *Tadpole.* |
|---|---|---|---|---|---|
| NaOH N/40 | 38 | <1 | <1 | 15 | 2 |
| Ether 5 per cent. | After 10 all revived | <1 | <1 | After 10 none revived | After 10 none revived |
| $HgCl_2$ 0.05 per cent. | 60 | <1 | <1 | 25 | 5 |

We notice in these tables that *Rhabditis* is about 2.5 times as resistant to N/40 NaOH, 0.05 per cent. mercuric chloride and N/100 HCl than is *Daphnia*, the next most resistant form. Tadpoles died more quickly in N/20 NaOH than in N/20 HCl. As shown in Table II., tadpoles died in 2 minutes in N/40 NaOH, while they died in about 4 minutes in N/30 HCl. This is probably due to the fact that the acid coagulates the body proteins as it penetrates, while the NaOH dissolves them. Tadpoles, which were placed in the 5 per cent. solution of ether for 5 minutes, revived though they were in a very bad condition. None of the *Rhabdites* revived after placing them 30 minutes in the solution of ether.

In order to study the penetration of an intra-vitam stain, some

*Rhabdites* were placed in a small quantity of neutral red solution on a slide, and were observed under a microscope. The stain entered through the mouth, and soaked out through the wall of the esophagus. In one worm the stain entered through the anus, but only a small region of the posterior part of the body was stained.

A solution of neutral red, which stained dead worms deeply, stained living worms only faintly; a strong solution, therefore, was used. Staining for 2.5 hours in such a strong solution, the anterior part of the body as faras the second bulb was deeply stained, but in the remaining part the intestine only was colored. The intestine was stained less deeply than the anterior regions of the body, and the stain became progressively lighter toward the posterior end. This seems to show that the stain entered through the mouth, but not through the cuticle nor through the anus. After staining for 20 hours the worms were still alive, and the anterior regions a little beyond the bulb of the esophagus were deeply stained. In the remaining part the intestine only was colored, and its posterior regions were stained less deeply than its anterior regions.

Such stained worms were placed in a weak solution of NaOH, and then the alkali entered through the mouth, and proceeded towards the interior. In about 20 minutes the portion of the body as far as the second bulb became yellow, but the change in color did not go further. Later a rapid diffusion of NaOH from the posterior end anteriorly was observed, and thus the entire body of the worm changed to yellow; the alkali seemed to enter through the anus. In another worm the change in colour proceeded anteriorly starting from the anus.

To show definitely that the cuticle prevents the entrance of the stain and the NaOH, the posterior regions of some worms were cut off. Then the stain entered through the mouth and the cut end. Such worms were, therefore, stained in toto for 4 hours, and at the end of this time they were still alive. Contrary to the case of uninjured worms the change in color carried by NaOH began at both ends, and proceeded faster anteriorly from the cut end than from the mouth. In about one hour and three quarters the change in color has been completed in such injured worms.

As in the case of neutral red, methylene blue entered through the mouth, and in no case penetrated through the cuticle. To stain the entire body of Rhabditis a strong solution was used for the same reason which has been stated in the case of neutral red. After staining 24 hours, some worms were still alive, but others were dead. The anterior part of the living worms as far as the second bulb was deeply stained, but the remaining part was lighter in colour.

Such stained worms were placed in 83 per cent. alcohol. The disappearance of the color began at the anterior end. The worms soon died in the alcohol, and the latter then entered through the anus. As far as the second bulb of the esophagus or a little beyond it, the disappearance of the color was due to the alcohol which entered through the mouth; in the remaining regions to that which entered through the anus. A small region of the anterior part of the intestine still remained stained after one hour in alcohol. In one worm I observed that the posterior division of the hermaphrodite reproductive organs was still stained, while the posterior region of the intestine which is found beside them was already reduced in color.

As in the case of neutral red, in some worms whose posterior ends had been cut off, the stain and alcohol entered through the mouth and the cut end.

## Summary.

1. *Anguillula aceti* is highly resistant to acetic acid. To see what is the case in other free-living nematodes *Rhabditis elegans* was used, and it was found that *Rhabditis* is much more resistant not only to various kinds of acids, but to other toxic substances also, as compared with the tadpole, *Daphnia*, *Aelosoma* and *Paramecium*.

2. When animals were stained in neutral red and in methylene blue, no case in which the stain entered through the cuticle was observed.

3. The entrance of NaOH and alcohol through the cuticle also could not be observed.

4. The stains, NaOH and alcohol entered through the mouth, and when the anus and vulva were opened, entered through them as well.

5. In the case of worms whose posterior ends had been cut off these substances in question entered through the cut end, as well as the mouth.

6. The conclusion to be drawn from these results seems to be that the impermeability of the cuticle is responsible for the resistance of *Rhabditis*.

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# BIOLOGICAL BULLETIN

## LUTEAL CELLS IN THE GONAD OF THE PHALAROPE.

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It has been shown by Pearl and Boring (1917, 1918) that there are in the ovary of the domestic fowl certain interstitial cells which are in many ways similar to, and which they claim are homologous with the corpus luteum in mammals. A thorough study has convinced the authors that these cells are a constant element in the ovary of all fowls but that they are not found in the testes of normal male fowls.

Boring and Morgan (1918) made a study of the gonads of both male and female Sebright Bantams, a race of fowls in which the males and females are feathered alike. They found in both ovary and testis groups of cells like those described by Pearl and Boring as luteal cells, and ascribed to these so-called luteal cells the function of influencing the type of feathering. Since luteal cells are found in the ovaries of normal females or hen-feathered fowls and not in the testes of normal males or cock-feathered birds, but in the testes of hen-feathered Sebright Bantams, they reason that the normal feathering of fowls would be of the male type were it not for the suppressing influence of the hormones secreted by the luteal cells. Further proof in favor of the contention is obtained from the well known facts of castration where hen-feathered females assume typical cock-feathers upon the removal of the ovary, and that the hen-feathered male Sebright becomes cock-feathered after the complete removal of the testes.

It has seemed that further evidence concerning the function of luteal cells might be brought to bear if a histological study were made of the gonads of birds other than fowls, and especially some bird in which the sex differences are reversed; that is some race of

birds in which the females are more brilliantly colored than the males. Such a sex difference is characteristic of the Phalarope. During the spring migration when the birds are in full breeding plumage the females are easily distinguished from the males by the more brilliant plumage of the back and neck. Bailey (1917) states the sex differences as follows: "Male in breeding plumage; upper parts dark plumbeous, striped on back with buff and black: sides of neck rufous, chest gray, upper throat and belly white. Female in breeding plumage; brighter colored, rufous extending across throat as well as on sides of neck." To one not acquainted with the facts concerning the sex differences of this group of birds the sexes would undoubtedly be mistaken. During the fall migration both males and females are lighter in color due to the loss of the rufous color and more nearly resemble one another.

In one respect the sex differences of the Phalarope do not compare with the sex differences of the fowls and that is in the fact that the color differences in the Phalarope are not associated with structural differences in the feathers, as is usually the case in the fowls. A microscopical examination of the feathers of the Phalarope showed that the color was due to pigment and not to a difference in structure. Whether or not this is an important difference and alters our problem can be determined only by a study of a large number of birds or different races.

The material with which this study has to deal consists of the gonads of both male and female Northern Phalarope, *Phalaropus lobatus*, Linn., collected during both fall and spring migrations along the Oregon coast. The tissues were taken from the birds as soon as possible after they had been shot, and were put into Bouin's fluid and left until it was convenient to care for them in the laboratory. Delafield's hæmatoxylin was found satisfactory and was used almost exclusively as a stain.

A study of the testes gave only negative results. In no case were there found any groups of cells resembling the characteristic packets of luteal cells found in the testes of the Sebright, and not even single cells which might be considered of that type. The intertubular material was not abundant and consisted of narrow spindle shaped cells with oval, more or less shrunken nuclei.

In the ovary, however, characteristic groups of the so-called luteal cells were found in the theca surrounding the oöcyte follicles

Figs. 1 and 2). These groups of cells are present in the material collected in May and September but the groups of cells seem to

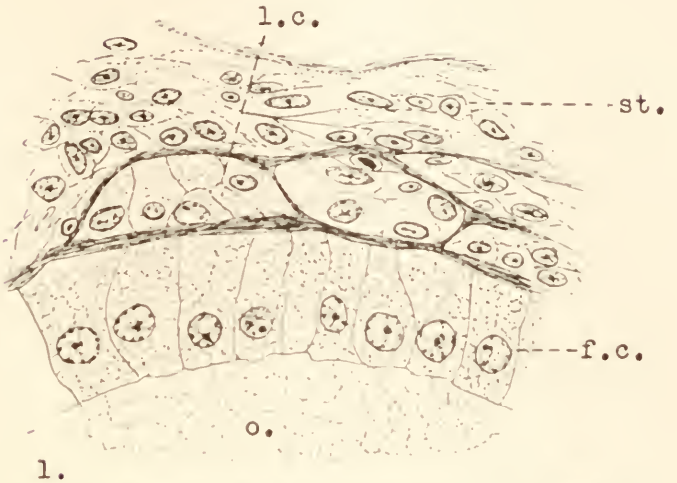


Fig. 1. Section of ovary of Phalarope in fall plumage. × 1000.

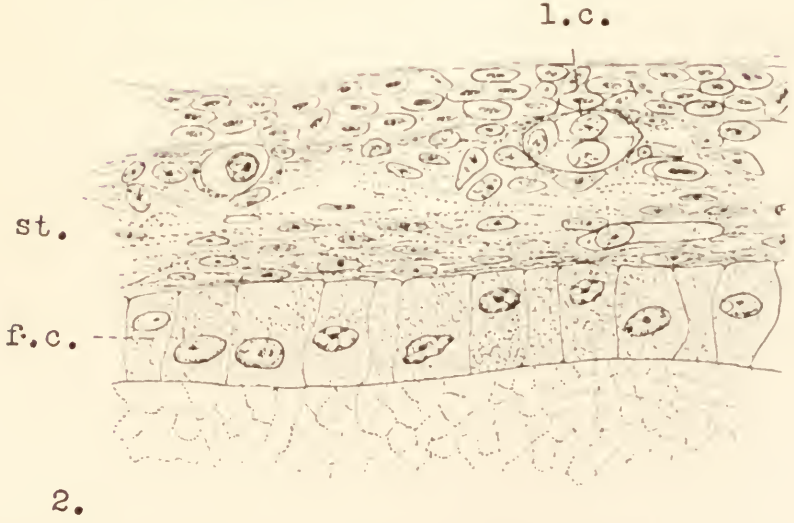


Fig. 2. Section of ovary of Phalarope in spring plumage. × 1000. *f. c.*, follicle cells, *l. c.*, luteal cells, *o.*, oöcyte, *st.*, stroma.

be somewhat larger and more clearly defined in the material collected in the fall when the sexes were more nearly alike as regards feathering. In no case could it be said that the groups of

luteal cells were numerous. In many sections there would be no packets at all while in others there would be one or two. In all cases where found the packets were clearly defined and easily demonstrable, being composed of cells having a relatively large amount of cytoplasm with rounded nuclei. The cytoplasm of the luteal cells takes very little of the hæmatoxylin stain. In this respect as well as in shape and size these cells differ from the cells of the stroma of the ovary.

The facts cited above do not indicate that luteal cells are in any way influential in suppressing color development in the Phalarope. In their consideration of the influence of the luteal cells on feathering in fowls Boring and Morgan have not separated the factors for color and structure in the feathers. In the present study only the color of the feathers is considered. The fact that luteal cells are found in the ovary while the female is so much more brilliantly colored seems to eliminate the possibility that the secretions of the luteal cells in any way suppress color development.

The evidence so far obtained is not sufficient to warrant any statement in regard to the problem of the association of color differences with structural differences in the feathers.

The recent work of Nonidez (1922) raises another question the solution of which will in a large measure depend on a more extensive study of the presence or absence of luteal cells in the gonads of birds. This author has shown that the so-called luteal cells as designated by Pearl and Boring and Boring and Morgan are in reality only the degenerating sex cords of the embryonic gonad. What have been termed luteal cells and what have been understood to be homologous with the corpus luteum of mammals, Nonidez has shown to have an entirely different origin and are therefore not to be confused in terminology with the mammalian tissues. Whether or not these remains of the sex cords may have an endocrine function is left by the author as an open question to be determined by a wider study of a variety of material. The facts as presented above as regards the presence of these cells in the gonads of the Phalarope do not seem to bring us any nearer a solution of the problem. There is no indication, in the Phalarope, that these groups of cells exert a suppressing influence on the development of the color of the feathers of the

two sexes. That they may have some other endocrine function, in the light of our present knowledge, is to be neither affirmed nor denied.

## Conclusions.

A histological study of the gonads of the Northern Phalarope shows that luteal cells such as are found in the gonads of Sebright Bantams are absent in the testes and present in the ovaries. Since the female is more brilliantly colored than the male there is no indication that these cells through an internal secretion influence the development of any differences in the color of feathers of the two sexes.

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# INTERACTIONS OF PROTOPLASMIC MASSES IN RELATION TO THE STUDY OF HEREDITY AND ENVIRONMENT IN *ARCELLA POLYPORA*.[1]

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## Introduction.

In the first article of this series, Kepner and Reynolds ('23) have shown that under favorable conditions *Difflugia* usually reappropriates, by fusion, fragments of protoplasm which have been severed from the cell-body. In the last paragraph of their summary these authors make the following statement: "Observations have been made in which individuals of the same species obtained from the same wild culture, showed a decidedly negative response towards each other's fragments, yet in other instances such cross-fusions did occur." Elsewhere in their paper two suggestions are made as to the probable explanation of why some specimens did fuse with fragments belonging to other individuals, viz: "(*a*) *The individuals were closely related by having a recent common ancestor.* (*b*) *By living in the same surroundings, the environmental influences have acted upon both organisms in such a way as to cause an identical physiological state.*" The question thus raised seemed to be of sufficient importance to justify further work on the subject; consequently the experiments incorporated in this paper were conducted for the purpose of clarifying the situation.

The previous publication was largely concerned with describing the process of protoplasmic fusion. Although a number of observations were made upon the reactions between cell-bodies and fragments from other individuals of the same species, no attempt was made to culture the organisms and then test for reactions between members of a clone. As a result, only two

[1] This is the second article of a series dealing with the phenomenon of fusion between protoplasmic masses. The first article was written by Wm. A. Kepner and B. D. Reynolds ('23) from the University of Virginia. Further work on the subject is now in progress.

types of reactions were observed: (*a*) fusion and (*b*) failure to attract, or if contact was made the fragment would be pushed along for a while, without causing any apparent disturbance, and then abandoned.

During the course of this investigation, involving much more extensive experiments, it was found that in some cases the masses would readily attract [1] each other, but upon making contact there would be a sudden contraction of the involved regions as if violently shocked, while the protoplasm constituting both the fragment and the organism's pseudopod (*s*) which had made contact with it would be shattered into bead-like masses. See Fig. 1: *A*, *B*, and *C*. This type of reaction was observed so frequently that it is referred to throughout this paper as the "shattering" reaction.

As the investigation progressed it seemed advisable to divide the problem into three sections; and to study the reactions between organisms and fragments from related specimens under the influence of three different conditions as follows:

I. Similar environments.
II. Different environments.
III. Identical environments.[2]

Numerous methods have been employed in studying variations among the protozoa, such as: rate of reproduction, measurements of size, counting the number of spines, teeth, tentacles, etc., resistance to poisons and to high temperatures. In this study, for the first time, advantage has been taken of the property possessed by certain organisms of fusing with their own or fragments from closely related specimens. The superiority of this method lies in the fact that it affords a quick and definite means of determining

[1] In this, as in the previous paper, it was not determined why two such masses were attracted to each other. When protoplasmic fragments were not present the organisms would move about at random; but when pieces of protoplasm which had been detached from themselves or from related individuals were within two or three hundred micra of them, they would invariably proceed directly towards the fragments. This statement is based on over fifteen hundred observations. A series of experiments are being planned to ascertain, if possible, the stimulus involved in these positive reactions.

[2] The organisms comprising the two lines under observation were placed in the same concavity, or else, portions of the culture media were frequently exchanged between the concavities containing them.

minute physiological differences—even before these changes have become expressed morphologically. Furthermore, the physiological difference between two organisms, as indicated by this method, appears to be more permanent and less subject to fluctuations than certain morphological variations which have been utilized by others. In some instances cross-fusion was observed to take place readily between specimens belonging to the same clone—though they appeared quite different to the eye. In other words: the physiological variations, as determined by means of the ability or inability of an individual to fuse with a fragment of protoplasm belonging to another, seem to be independent of differences in size or shape of the two animals concerned.

Fusion between cell-bodies and severed fragments has been observed by the author in ten species of the genus *Difflugia*, in *Arcella polypora*, *Centropyxis aculeata*, and also other forms of protozoa. The phenomenon seems to be fairly common among the thecamœbæ. Perhaps it would be well to emphasize at this point the fact that throughout these experiments any individual *Arcella* would fuse with its own fragments (provided they were not too far removed, or had not been separated for too long a period) under any environmental conditions to which they were subjected.

Acknowledgments.

This problem suggested itself to me while I was discussing with Professor Wm. A. Kepner, the results of our work ('23) *Reactions between Cell-bodies and Pseudopodial Fragments of Difflugia*. I wish to express my deepest gratitude to Professor R. W. Hegner and Dr. Wm. H. Taliaferro, under whose direction the work was done, for their valuable advice and criticisms. I am also deeply indebted to Professor H. S. Jennings for reading the manuscript and making some important suggestions.

## The Organism.

The experimental animal used in this investigation was a multinucleated *Arcella* closely resembling the species described by Penard ('85) as *A. polypora*. Hegner ('20) worked with the same organism, calling it *A. polypora*. However, in the same paper he also gave measurements of a larger multinucleated form which

agrees with the amended description of *A. polypora* given by Penard ('92) viz: "*très grande, à bouche très largement ouverte, et toujours pourvue de noyaux en nombre considerable.*" Apparently Hegner is the only investigator in this country who has reported the existence of *Arcellae* containing many nuclei. Leidy ('79) states that he was able to detect two, occasionally one, and rarely three, though admitting that he had possibly overlooked the presence of some nuclei in the larger forms of *Arcella*. Occasionally large, polynucleated, wide-mouthed specimens were found in my collections. The differences between these two multinucleated forms seem to be great enough to justify the creation of a new species. However, if this is done, the law of priority makes it necessary to retain the name *A. polypora* for the smaller form which was used in these experiments.

Because of the existing confusion it is deemed advisable to say a few words regarding certain characteristics possessed by the organism studied: shape, structure, and coloration of shell similar to *A. discoides;* diameter of shell from 80 to 120 micra; height of shell from 25 to 40 micra; oral orifice approximately equivalent to one third the diameter of shell; number of nuclei usually eight or ten. Careful scrutiny under the oil immersion failed to reveal any minute pores around the mouth of the shell, but the shadows at the outer margins of the large cancelli lining the buccal aperture might readily be misinterpreted as such under a low magnification.

*Arcella polypora* was selected as the object for study because it reproduces rapidly, is easy to culture, and is capable of withstanding the influences of various stimuli. This rhizopod may be found in either large or small bodies of water, living especially on the under surfaces of aquatic plants, in decaying vegetable matter, and ooze. On April 10th. 1922 a collection, containing many *A. polypora*, was obtained from an artificial pool in the botanical garden near the Johns Hopkins Biological Laboratory. All of the organisms used in these experiments were descendants of this lot. A mass culture was kept going, and as new individuals were needed they were isolated and new clones were started from them.

## Methods.

### (*a*) *Apparatus and Technique.*

In carrying out these experiments more standardized and exact methods were employed than those used in the first article of this series, though they were essentially the same. In every case (unless specified) distilled water (pH = 0.7) was used, and each animal was washed once or twice before being transferred to the slide on which the amputations were made. Furthermore, any detritus clinging to the shell was removed before the operation. In all of the cross-fusion experiments the two organisms to be tested were placed on the same slide, and when a fragment was severed from one, that individual was moved away and the other placed near the fragment of protoplasm. The glass needles, with which the protoplasmic masses were detached, were drawn from standard Pyrex tubing $\frac{1}{4}$ OD furnished by the Corning Glass Company, Corning, N. Y. All operations were performed under the compound microscope—32 mm. objective and No. 10 eyepiece (B. & L.)—while the 16 mm. objective was used in making the observations. A camera lucida was attached to the microscope and at intervals (usually one minute) the outlines of the objects were traced with a pencil. These sketches were 170 times the size of the objects.

The pseudopods of *A. polypora* are usually digitate and rarely extend far beyond the periphery of the shell, making it very difficult to sever masses of protoplasm by cutting them off with a needle as was done in the case of *Difflugia*. This difficulty was overcome by taking advantage of two things—(*a*) the shape of the shell, and (*b*) the fact that if undisturbed for a while the animal ordinarily attaches itself to the substratum. The organism is shaped somewhat like a shallow bowl, the outer rim of the shell forming a rather sharp edge, Fig. 2. If, after a pseudopod has been extended beyond the outer margin, pressure is applied to the top of the shell the pseudopod will be caught between the rim and the sub-stratum and its distal end will thus be pinched off. Severance is aided by the animal's sudden reaction to this stimulus. Sometimes a specimen would fail to protrude pseudopods of sufficient length to permit use of the method just described; consequently it was left undisturbed for a few min-

utes and then suddenly displaced by means of a glass filament. This procedure would frequently result in a large mass of protoplasm being torn from the organism.

### (*b*) *Culture.*

Hollow-ground slides, each containing two concavities, were used as receptacles in cultivating the organisms. Seven drops of the desired culture medium were put into each concavity and the animals were transferred to them. After this the slides were labelled and placed in transparent glass moist chambers which were kept before a window, unless otherwise stated.

At first the method described by Jennings ('16) for *Difflugia* was used in preparing the culture medium, *i.e.*, a quantity of ooze from a pond in which the organisms were found to be living was shaken up in water, this was allowed to settle, and then the water was decanted off and used. Although other investigators have obtained successful results by using culture media prepared in this way, several objections to its use in these experiments were evident: first, fresh material was hard to obtain, since no ponds were near the laboratory in which the work was done; secondly, a constant culture medium was not insured, for the plant and animal life in a given pond vary constantly; and finally, it was necessary ever to be on guard against possible contamination with wild specimens and injurious chemical agents. After encountering these difficulties for two months the above method was discarded in favor of a hay infusion culture medium which dispensed with all of the objections mentioned, and in which the organisms thrived even better. It was prepared as follows: 10 grams of clean timothy hay was placed in a clean beaker containing 250 c.c. of neutral distilled water. This was boiled slowly for five minutes, then strained through two thicknesses of cheese cloth, after which it was stored, in quantities of about 3 cc., in small sterile test tubes. The tubes were then plugged with cotton and placed in boiling water for fifteen minutes. Two days later they were subjected to boiling water again for the same period of time, in order to kill any bacteria which might have resisted the first sterilization by being in the spore stage. The liquid in the tubes constituted what was known as stock solution; it would keep for months without deteriorating

provided evaporation didn't take place. In making up the culture medium one part of the stock solution was taken and to it were added nine parts of distilled water—giving a 10 per cent hay infusion solution. When other ingredients such as sugar, alcohol, etc., were used, they were mixed with distilled water in the desired proportions and then nine parts of this were added to one part of the stock solution. Thus, in every case the percentage of hay infusion remained the same. After a tube containing some of the stock solution had been opened and a part of its contents used the remainder was discarded. The *Arcellæ* grown in this medium subsisted largely on bacteria, the progenitors of which were introduced with the organisms.

### (*c*) *Pedigrees.*

"Pure" lines, or clones, were obtained by isolating specimens from the wild culture and designating them, for example, as the progenitors of clone *A*, clone *B*, etc. Upon dividing, the daughter-cell remaining in the old shell, and all of its descendents, were referred to as clone *A* line *a*, while the daughter-cell occupying the new shell, and all of its descendents, were denominated clone *A* line *ad*. Fig. 3 expresses graphically the procedure followed.

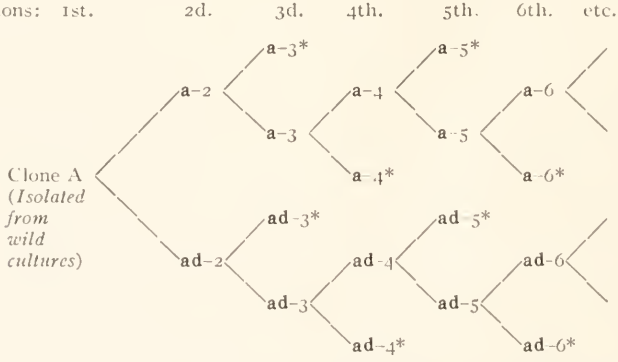


FIG. 3. Diagram illustrating method employed in recording the relationship of experimental animals. Those marked with an asterisk were either used for observations, or else discarded.

When a different environment was imposed on one line of a clone that line was designated, for instance, as clone *A* line **a**-*su* (*su* indicating that sucrose was added to the culture medium in

which this line was nourished). Ordinarily lines were perpetuated through the individuals remaining in the old shells, but this rule was not strictly adhered to since experience showed no difference in the results obtained when other members were used. From two to four individuals of each line were kept in culture as a precaution against possible fatalities.

### I. Similar Environments.

These experiments are discussed under the heading "similar" environments because the external conditions surrounding the two lines of the clones involved were as nearly alike as it was possible for the experimenter to make them. Though kept apart from the first division of the clonal parent, the two diverging lines were treated in exactly the same manner; for instance: the same pipette was used in transferring members to fresh concavities; these concavities contained culture medium taken from the same bottle; afterwards the depression slides containing the organisms were placed alongside each other in the same moist chamber. Frequent tests were made, according to the method described above, between individuals of one line and fragments of protoplasm from individuals of the other. It was found that daughter-cells (members of the second generation) would readily fuse with each other's severed fragments — and that such reactions would occur regardless of whether or not the organism taking up the fragment had just previously lost a part of its own protoplasm. Furthermore, in every case when both lines were kept under similar conditions, cross-fusions continued to be exhibited for fifteen or more generations; consequently in later experiments tests for interactions were not begun until several generations had elapsed.

A series of experiments conducted between the two lines of clone I are given in extenso in Table I. It will be seen by referring to this table that cross-fusion between line i and line id persisted until the 22d day and 24th generation. After this no further inter-fusions occurred, but instead the protoplasms usually were shattered upon making contact. Attention should be called to the fact that in some of the experiments listed in Table I. the organism was on its back when placed near the fragment; in other cases the fragment would become detached from the sub-

## TABLE I.

INTERACTIONS OF CELL-BODIES FROM ONE LINE AND PSEUDOPODIAL FRAGMENTS OF SPECIMENS FROM THE OTHER IN CLONE I, LINES i AND id. BOTH LINES WERE EXPOSED TO SIMILAR ENVIRONMENTS. 10 PER CENT. HAY INFUSION USED AS CULTURE MEDIUM. EXPERIMENTS WERE STARTED ON JUNE 25, AND ENDED ON JULY 31, 1922.

| No. of Days Since Experiment was Begun. | Generation of Cell-body. | Generation of Fragment. | Horizontal Dimensions of Fragment in Micra. | Distance between Cell-body and Fragment: in Micra. | Time in Minutes before Contact was Made. | Reaction after Contact. |
|---|---|---|---|---|---|---|
| 16 | i-18 | id-17 | 9 x 25 | 50 | 2 | Fusion within 1′. |
| 16 | id-17 | i-18 | 10 x 18 | 100 | 3 | Immediate fusion. |
| 16 | i-18 | id-17 | 15 x 40 | 110 | 3 | Fusion after 3′. |
| 18 | i-20 | id-19 | 12 x 15 | 90 | – | No contact. |
| 18 | id-19 | i-20 | 10 x 48 | 85 | 1.5 | Fusion within 30″. |
| 22 | i-24 | id-24 | 30 x 40 | 100 | 4 | No fusion. |
| 22 | id-24 | i-24 | 9 x 30 | 150 | 3 | Violent contraction. |
| 22 | id-24 | i-24 | 12 x 25 | 55 | 2 | Shattering reaction. |
| 25 | i-28 | id-27 | 8 x 38 | 95 | – | No contact. |
| 25 | i-28 | id-27 | 7 x 45 | 48 | – | No contact. |
| 25 | id-27 | i-28 | 45 x 55 | 32 | 1 | Shattering reaction. |
| 27 | id-28 | i-31 | 10 x 40 | 10 | 0.5 | Shattering reaction. |
| 29 | i-34 | id-32 | 14 x 20 | 82 | 2 | No fusion. |
| 29 | id-32 | i-34 | 12 x 30 | 15 | 3 | Shattering reaction. |
| 29 | id-32 | i-34 | 25 x 35 | 6 | 3 | Shattering reaction. |
| 32 | i-38 | id-36 | 20 x 30 | 62 | – | No contact. |
| 32 | i-38 | id-36 | 20 x 30 | 0 | 0 | Slight shattering. |
| 32 | id-36 | i-38 | 24 x 26 | 40 | 2 | Slight shattering. |
| 32 | i-38 | id-36 | 10 x 60 | 10 | 6 | No fusion. |
| 32 | id-36 | i-38 | 15 x 45 | 47 | 1.5 | Shattering reaction. |
| 32 | i-38 | id-36 | 8 x 25 | 0 | 0 | No fusion. |
| 36 | i-43 | id-41 | 7 x 70 | 78 | 2 | Shattering reaction. |
| 36 | i-43 | id-41 | 6 x 40 | 105 | 4 | Shattering reaction. |
| 36 | id-41 | i-43 | 20 x 60 | 53 | 1 | Shattering reaction. |

stratum and float away, or else be pushed along by the organism's pseudopods without making intimate contact with them. This accounts, to a great extent, for discrepancies observed in the time required to make contact, and also for the cases marked *No fusion* and *No contact.*

A similar table might have been arranged for any of the other clones studied in this manner, but to take up so much space with tables would be both impracticable and unnecessary; therefore a single table has been prepared which gives the number of days and generations elapsing before the intraclonal reactions became negative (as indicated by shattering instead of fusing of the pro-

toplasms). This table (Table II.) shows a range of from 18 to 27 generations, and from 15 to 32 days, in the time of the first appearance of negative reactions between two lines of a clone, or an average of 22.25 generations and 21.58 days. After two lines had once become negative to each other no further cross-fusions took place between them unless certain modifications were made, the nature of which will be discussed later.

## Table II.

Table Showing Date and Generation of First Exhibition of Shattering Reaction in Twelve Clones of Arcella. Both Lines of these Clones were Exposed to Similar Conditions.

| Experiments Begun. | Clone. | Culture Medium used. | First Neg. Reaction Observed. | | | No. of Observations Made. |
|---|---|---|---|---|---|---|
| | | | No. of Generations. | | Days Since Beginning. | |
| | | | 1st. Line. | 2d. Line. | | |
| May 5 | *A* | Pond water | 22 | 22 | 24 | 15 |
| " 5 | *B* | " " | 21 | 21 | 21 | 38 |
| " 10 | *C*[1] | " " | — | — | — | 3 |
| " 5 | *D*[2] | " " | — | — | — | 10 |
| June 1 | *E* | 25% hay infu. | 23 | 27 | 32 | 25 |
| " 1 | *F* | Pond and Hay | 23 | 23 | 30 | 37 |
| " 25 | *I*[3] | 10% hay infu. | 24 | 24 | 22 | 24 |
| " 25 | *J* | " " " | 18 | 18 | 15 | 39 |
| " 25 | *K* | " " " | 25 | 25 | 18 | 37 |
| " 25 | *L* | " " " | 20 | 20 | 17 | 30 |
| " 25 | *O* | " " " | 18 | 18 | 16 | 16 |
| " 25 | *P* | " " " | 19 | 20 | 18 | 21 |
| Aug. 29 | *Q* | " " " | 26 | 26 | 23 | 8 |
| " 29 | *R* | " " " | 25 | 24 | 23 | 18 |
| | | Average: 22.25 gen. | | | 21.58 days. | |

Before these cultures were discarded the culture medium, in which the organisms were living, was tested for its pH value by means of the Phenolsulphonephthalein indicator. In every case it was found to be neutral; *i.e.*, no detectable change had taken place because of the presence of the organisms.

## Summary of Section I.

In summing up the results obtained from these experiments, the evidence indicates that physiological differences do develop

[1] Cross-fusion obtained on the 16th day. Cultures died before further observations were made.

[2] Cross-fusion obtained on the 10th day—cultures died.

[3] This set of experiments is given in extenso in Table I.

among the members of a clone, without necessarily being accompanied by corresponding morphological differences, and that these changes can be demonstrated by the cross-fusion method within relatively short periods of time—approximately 22 days. Since both lines of a given clone were subjected to the same external influences, the inference might be drawn that such protoplasmic changes are probably due to inherited variations and not to the action of environmental factors. However, later experiments show that this position is untenable.

## II. Different Environments.

Having determined the time required for intra-clonal differentiation under similar environments, it was now possible to subject the two lines of a clone to different environments and observe the relative effects such treatment would have on their behavior towards each other's fragments. In carrying out these experiments such factors were employed as might occur in nature, viz: (*a*) 25 per cent hay infusion, (*b*) sucrose, (*c*) saline, (*d*) acetic acid, (*e*) sodium carbonate, (*f*) alcohol, (*g*) darkness, and (*h*) temperature. The organisms seemed to thrive best in 10 per cent hay infusion, and since this could be prepared by a given formula, it was used in every case (except one) as the standard culture medium: *i.e.*, one line of a clone was grown in it at room temperature in front of a window, while the other was altered as desired.

### (*a*) *25 Per Cent Hay Infusion.*

An organism was isolated from a wild culture and placed in a concavity containing pond water. Upon dividing, the new cell thus formed was transferred to a receptacle containing 25 per cent hay infusion and designated as clone H line **h**-*hay*, while the individual remaining in the old shell was placed in a concavity containing fresh pond water and now became known as clone H line **h**. These two daughter-cells were then placed in the same moist chamber, so that the only difference in their environments was represented in the culture media. The usual tests for cross-fusion between the lines were made with the following results: on the second day after the beginning of the experiments +, 4th +, 6th +, 8th — o, 9th — — — — o — .[1]

[1] The significance of these symbols is as follows: + fusion occurred; — shatter-

A similar series of experiments were conducted between two diverging lines of clone W. In this case seven observations were made, extending through the eleventh generation, with the result that cross-fusion took place in every instance. Further observations were made impossible on account of death in one of the lines. In one case then, the time required for two related lines to exhibit the negative reaction was greatly reduced, while in the other death interfered with the determination of this point. If standing alone, further evidence would certainly be necessary before much emphasis could be placed on the significance of these observations; but, since the conclusions drawn, from the experiments dealing with different environments, were largely deducted from later observations, it was decided to include these as they stand without attempting to add more convincing proof.

(*b*) *10 Per Cent. Hay Infusion Containing 1 Per Cent. Sucrose.*

In these, and all succeeding experiments, the *standard* culture medium was 10 per cent. hay infusion. After the first division of the clonal parent, one of the daughter-cells was transferred to a concavity containing 10 per cent. hay infusion plus 1 per cent. sucrose, while the other was kept in the standard culture medium. In every other respect the two lines arising from these individuals were subjected to similar influences. In order to avoid possible contaminations, a different pipette was used in transferring members of these two lines to fresh culture media. The density of the medium was, of course, increased upon the addition of sugar, which resulted in the newly formed shells becoming smaller and smaller, so that it was considered advisable (perhaps necessary) to remove the osmotic influence after six or seven days; consequently, after having remained in the medium containing 1 per cent. sucrose for approximately that length of time, the organisms were put back into 10 per cent. hay infusion. From this time on both lines of a clone were living amidst the same surroundings. It was found that cross-fusion between the two lines continued for five or six days, then for a period of six or seven days the interactions were negative (even though the osmotic agent had

ing reaction; o contact made but neither fusion nor shattering followed. The number of symbols placed after a figure indicates the number of observations made on that day.

been removed) after which another short period of inter-fusion ensued. This is clearly shown in Table III.

## Table III.

Showing Interactions of Related Lines, k and k–*su*. The Environment was the Same for Both Lines, with the Exception that for 7 Days Line k–*su* was Kept in a 1 Per Cent. Sucrose Solution. The Experiments were Begun on June 27, and Discontinued on July 22, 1922.

| No. of Days after Beginning of Experiment. | Generation of Cell-body. | Generation of Fragment. | Horizontal Dimensions of Fragment in Micra. | Distance between Cell-body and Fragment in Micra. | Time in Minutes before Contact was Made. | Reaction after Contact. |
|---|---|---|---|---|---|---|
| 2 | k–*su*–3 | k–2 | 9 x 34 | 75 | 10 | Immediate fusion. |
| 2 | k–2 | k–*su*–3 | 12 x 18 | 40 | 2 | Fusion within 15″. |
| 3 | k–4 | k–*su*–4 | 12 x 40 | 93 | 4 | Fusion after 11′. |
| 3 | k–*su*–4 | k–4 | 8 x 24 | 63 | 2 | Fusion within 15″. |
| 3 | k–*su*–4 | k–4 | 9 x 33 | 67 | 2.5 | Fusion within 15″. |
| 3 | k–*su*–4 | k–4 | 8 x 20 | 112 | 3 | Fusion within 30″. |
| 4 | k–6 | k–*su*–5 | 15 x 80 | 45 | 3 | Immediate fusion. |
| 4 | k–6 | k–*su*–5 | 8 x 35 | 60 | 3.5 | Fusion within 30″. |
| 4 | k–*su*–5 | k–6 | 8 x 45 | 0 | 0 | Fusion after 2′. |
| 6 | k–8 | k–*su*–6[1] | 35 x 75 | 95 | 3 | Shattering reaction. |
| 8 | k–9 | k–*su*–7[2] | 12 x 30 | 9 | 1 | Shattering reaction. |
| 9 | k–10 | k–*su*–9[3] | 27 x 80 | 95 | 2 | Shattering reaction. |
| 9 | k–*su*–9 | k–10 | 12 x 15 | 55 | – | No contact. |
| 11 | k–*su*–11 | k–13 | 9 x 25 | 85 | 1 | Shattering reaction. |
| 11 | k–13 | k–*su*–11 | 8 x 14 | 32 | 0.5 | No fusion. |
| 13 | k–16 | k–*su*–14 | 24 x 26 | 26 | 2 | No fusion. |
| 13 | k–*su*–14 | k–16 | 10 x 45 | 13 | 1 | Immediate fusion. |
| 13 | k–*su*–14 | k–16 | 8 x 40 | 90 | 2 | Immediate fusion. |
| 13 | k–16 | k–*su*–14 | 9 x 32 | 63 | 1 | Immediate fusion. |
| 13 | k–16 | k–*su*–14 | 8 x 30 | 70 | 1 | Immediate fusion. |
| 13 | k–*su*–14 | k–16 | 25 x 30 | 40 | 2 | Fusion within 1′. |
| 13 | k–16 | k–*su*–14 | 10 x 56 | 85 | 2 | Fusion within 1′. |
| 14 | k–17 | k–*su*–15 | 12 x 30 | 140 | 4 | No fusion. |
| 15 | k–19 | k–*su*–16 | 10 x 20 | 115 | 2 | Immediate fusion. |
| 15 | k–*su*–16 | k–19 | 8 x 40 | 90 | – | No contact. |
| 20 | k–*su*–21 | k–24 | 25 x 60 | 37 | 4.5 | Shattering reaction. |
| 23 | k–28 | k–*su*–25 | 9 x 50 | 10 | 1 | Shattering reaction. |
| 23 | k–*su*–25 | k–28 | 8 x 10 | 33 | 1 | Shattering reaction. |
| 25 | k–*su*–28 | k–31 | 12 x 30 | 62 | 1 | Shattering reaction. |
| 25 | k–31 | k–*su*–28 | 8 x 25 | 35 | 1 | Shattering reaction. |

[1] Later specimen k–*su*–6 fused with its own fragment which had caused a shattering of specimen k–8's cytoplasm.

[2] The young individuals of line k–*su*, having been subjected to the influence of 1 per cent. sucrose for 7 days, were reduced in size to a diameter of 65 micra (the normal diameter being 100 micra). On this day line k–*su* was put back into 10 per cent. hay infusion, where it remained during the rest of the experiments.

[3] Later specimen k–*su*–9 fused with its own fragment after it had been abandoned by specimen k–10.

By referring to Table III. it will be seen that cross-fusion between the two lines persisted through generations k–*su*–5 and k–6, while two days later (k–*su*–6 and k–8) their potency to fuse with the other's fragments had ceased. On the 2d day after the first negative reaction was observed, line k–*su* was taken out of the sugar solution and placed in the same medium used for line k; nevertheless, the reactions between the two lines continued to be of the shattering type for 4 more days; *i.e.*, cross-fusion was not observed again until the fifth day after the two lines had been restored to a common environment (k–*su*–14 and k–16). For the next few days the inter-reactions were positive, but from generations k–*su*–21 and k–24 until the experiments were discontinued, shattering of the protoplasms took place in every instance. Compare the second shattering period with results shown in Table II.

An interesting point brought out by this study, is the fact that though the normal size was regained two days after the individuals of line k–*su* were removed from the sucrose solution, the inability to cross-fuse with fragments of specimens from line k persisted for five days. In other similar experiments the stability of this physiological variation as compared to the morphological difference in size was shown even more strikingly.

Three other series of experiments, in which 1 per cent. sucrose was used as the modifying factor, were conducted, and all of them gave results parallel to those shown in Table III. For instance, on June 7th the progenitor of clone M was isolated from a wild culture and upon dividing the next day, lines m and m–*su* were started. Cross-fusion between these two lines was observed on the third day (generations m–4 and m–*su*–4), shattering reaction exhibited on the 6th (m–7 and m–*su*–6), line m–*su* put back in standard culture medium on the 7th, negative inter-actions persisted until the 15th (m–18 and m–*su*–15), then cross-fusions took place during the next five days, all of the interactions after this were of the shattering type. Twenty observations were made in this series of experiments.

Observations in clone J, lines j and j–*su* were begun on August 27th. Cross-fusion between line j and line j–*su* on the 4th day (j–4 and j–*su*–5), negative on the 6th. (j–6 and j–*su*–7), line j–*su* removed from 1 per cent. sucrose on 6th, negative inter-

actions persisted until the 15th (**j**-16 and **j**-*su*-16); this was followed by a brief period of cross-fusion, after which the interactions became permanently negative—number of observations in this case was 35.

A similar series of experiments between lines **n** and **n**-*su* of clone N were started on August 27th. In this case line **n**-*su* was removed from the sugar solution before the negative condition had been attained; consequently cross-fusion continued until the 15th day. From this time on all of the interactions were negative. Forty observations were made in this series of experiments.

### (*c*) *10 Per Cent. Hay Infusion Containing N/10 Saline.*

Two series of experiments were conducted in which one line of a clone was placed in a culture medium of 10 per cent. hay infusion plus one tenth normal saline solution, while the other line was kept in the standard medium—the only difference between the environments of the two lines was that the culture medium used for one contained 0.09 per cent. salt. During the first three days the organisms in the saline solution seemed to grow and reproduce normally; after this, three days elapsed before another division occurred; then further growth ceased. In the meantime tests showed that cross-fusion between the two lines took place readily. After keeping the organisms in saline solution for eight days they were put back into standard culture medium. The interactions continued to be positive for two more days, then the experiments were discontinued.

The evidence obtained from these observations indicates that N/10 saline is an unfavorable environment for *A. polypora;* but regardless of this, no physiological change that can be detected by the cross-fusion method was thus produced.

### (*d*) *10 Per Cent. Hay Infusion Containing Acetic Acid.*

Numerous attempts were made to culture the organisms in 10 per cent. hay infusion to which had been added various amounts of acetic acid, but after repeated efforts it was found impossible to keep the animals alive in solutions containing only 0.01 per cent. acetic acid. Other acids, or weaker concentrations of acetic acid, were not tried.

### (*e*) *10 Per Cent Hay Infusion Containing 0.025 Per Cent Sodium Carbonate.*

By beginning with one per cent. sodium carbonate and each time decreasing the proportion, it was determined that *A. polypora* could be successfully grown in a medium containing 0.025 per cent. of this substance. Only one set of experiments was conducted, but the results obtained were very definite. On June 29th an individual was taken from clone K and set aside to become the progenitor of a new clone S. The next day it had divided, so one of the daughter-cells resulting from this division was placed in 10 per cent. hay infusion containing 0.025 per cent, sodium carbonate, while the other was kept in standard medium. The size, activity, and rate of reproduction of the organisms subjected to the influences of sodium carbonate (a weak base) seemed to be perfectly normal. Results of the reactions between line S and line S–*s.c.* are given by means of symbols (See footnote page 116 for meanings). On the second day after the experiments were begun the interactions were + +, 4th o o +, 6th line S–*s.c.* permanently transferred to standard medium, 8th − −, 9th + +, 11th + +, 14th − o, 19th −, 21st − − 23d − −. No further observations were made after this date. These results strongly suggest that, if one line of a clone be placed in 10 per cent. hay infusion and the other line in a similar medium to which 0.025 per cent. sodium carbonate has been added, the physiological constitutions of the members comprising the two lines will quickly become so differentiated that cross-fusion will not take place between individuals belonging to one line and fragments of specimens from the other. Such changes are shown to persist for some time after the causative agent has been removed.

### (*f*) *10 Per Cent. Hay Infusion Containing 1 Per Cent. Alcohol.*

On August 28th specimens were taken from two clones, which had been under observation since June 25th, and with them two sub-clones, J and K, were started. These sub-clones were divided into two lines each; one of which was kept in standard medium, while the other was placed in 10 per cent. hay infusion plus 1 per cent. alcohol—the latter being designated sub-clone

J line **j**–*a*, and sub-clone K line **k**–*a* respectively. The organisms subjected to alcohol seemed to thrive quite well—the rate of fission, in both lines being slightly greater than that of those kept in standard medium. It was found, by testing the interactions of related specimens grown in these different environments, that the results were parallel in both cases; yet different from those obtained when substances other than alcohol were used for altering the environmental conditions. These observations were extended over a period of 65 days, representing 66 generations in sub-clone J and 63 in sub-clone K. The results are indicated by symbols (see footnote on page 116 for meanings). For sub-clone J, reactions between line **j** and line **j**–*a* were as follows: on the 3d day + o, 5th + o, 8th − − − −, 10th + +, 14th − − o −, 16th − o, 18th − o o, 21st + +, 23d + + +, 29th o o −, 33d + + +, 35th − −, 46th − o − −, 63d − −, 65th − o.

For cub-clone K, the reactions between line **k** and line **k**–*a* were as follows: on the 3d day o +, 5th + o, 7th +, 8th + + + +, 9th − − − −, 10th + + +, 11th + + +, 14th − −, 16th + o + o, 18th + +, 21st + + + + +, 23d + +, 28th − o o o, 30th + +, 35th + +, 46th o + +, 52d + +, 63d − −, 65th −.

As can be seen by glancing at these data, the interactions were variable and inconsistent. This suggests, perhaps, a fluctuating change of the protoplasm in contradistinction to the uniform conditions found to prevail in other experiments included in this section. No explanation of this is offered, since time did not permit an exhaustive investigation of the subject to be made; but it seems that one would hardly be justified in attributing the cause to a detrimental environment, for, as has already been dointed out, growth was not diminished by the presence of alcohol. Hegner ('19), found that *Arcella* dentata would grow and reproduce in a medium containing from 0.25 to 1 per cent. alcohol. However, the fission rate was retarded and irregularities in the shells of the offsprings were observed.

### (*g*) *One Line of a Clone Kept in Darkness, the Other Exposed to Light.*

Having found that related specimens could readily be made negative to each other's protoplasmic fragments by adding various substances to the culture medium in which one line was grown, the following question arose: *Are the changes thus brought about due to the direct action of the added material, or to some other causes?* With this in mind, a series of experiments was begun in which the same culture medium was used for both lines of a clone, and in every other way they were treated alike, except one line was placed in the dark while the other was kept before a window. Thus, in one line the organisms were exposed to light during the day, while light was entirely excluded from those comprising the other, except for the short time required to transfer them to new depression slides (a period of about two minutes every other day). Four experiments of this type were conducted, involving clones G, T, U, and V. In three out of the four cases, the rate of reproduction was greater among the organisms kept in darkness than among their relatives which were exposed to light. However, it is possible that this was due to a greater abundance of certain forms of bacteria upon which the *Arcellæ* fed; for it was observed that the bacterial growth was usually very vigorous in the cultures which had been kept in the dark.

The results obtained in these experiments may be briefly given as follows: In clone G, line **g** and line **g**-*d* continued to inter-fuse with each other's protoplasm until the 15th day (16th generation); in clone T, the first negative reaction was observed on the 12th day (12th generation); in clone U, shattering reactions occurred between line **u** and line **u**-*d* on the 13th day (15th generation); in clone V, cross-fusion between line **v** and line **v**-*d* ceased on the 13th day (15th generation). After two related lines had once become negative, no further cross-fusions occurred.

In these experiments the average time required for two lines of a clone to become so differentiated as to shatter each other's detached fragments of protoplasm was 13.25 days, and 14.5 generations. While this is a longer period than was required for the shattering reaction to be exhibited when sucrose, for instance, was used as the differentiating factor, it is 8.33 days

(and 7.75 generations) shorter than was the case when no change was made in the environments. It is thus shown that the absence of light does affect, either directly or indirectly, the physiological constitution of *A. polypora*.

(*h*) *The Two Lines of a Clone Exposed to Different Temperatures.*

Specimens were exposed for twenty-four hours to temperatures of 36, 35, 30, 28, and 27 degrees centigrade, but in every case death occurred during the period of incubation. However, if incubated for only four hours, the organisms were able to resist a temperature of 36 degrees C. Tests were made to determine if such short exposures to heat would affect the protoplasm enough for it to be detected by the cross-fusion method. No changes of this sort could be revealed; regardless of whether the observations were made immediately after incubation, or twenty-four hours later.

Attempts to culture *A. polypora* at low temperatures proved to be more successful. Fifteen degrees centigrade was arbitrarily chosen as the low temperature environment, while room temperature (21 degrees C.) was used as the norm. A lower temperature was not selected because it was desirable that the organisms reproduce at a fairly rapid rate; though at 15 degrees C. the fission rate was reduced approximately one half.

On Nov. 7 two temperature experiments were started, involving Clone X line **x** and line **x**–*t*, and clone Y lines **y** and **y**–*t*. The same culture medium was used for both lines of a clone, and the lines which were not kept in the low temperature incubator were put in a darkened place to counteract any effect lack of light might have had on the organisms in the refrigerator. As usual, tests were made at frequent intervals to determine the interactions of two diverging lines. The results obtained are shown below by means of symbols.[1] In clone X—reactions between line **x** and line **x**–*t* on the 6th day + ¢ + 0, 10th + ¢, 14th − − ¢ ¢, 16th 0 0, 17th ¢ − −, 19th − ¢ ¢.

In clone Y—reactions between line **y** and **y**–*t* on the 6th day ¢ − +, 8th + + +, 10th + +, 12th + + +, 14th + + +, 16th + 0 +, 17th 0 −, 19th − −.

[1] + signifies that fusion took place; − shattering reaction; 0 contact was made but neither fusion nor shattering followed; ¢ contact was not made. The number of symbols placed after a figure indicates the number of observations made on that day.

If the one shattering reaction observed on the 6th day of the experiment between the two lines of clone Y be disregarded, the above data will show that negative interactions first occurred on the 14th day in clone X, and on the 17th day in clone Y. Taking the higher figure 17 it is 4.58 earlier than the average time required when the environments in the two lines of a clone were similar. However, if we consider the number of generations, a greater difference is shown. On the day the first negative reaction was observed in clone X, line x had produced 15 generations and line x + 8, which if added and the result divided by 2 yields 11.5 generations, while in clone Y, line y represented 18 generations and line y + 10, or an average of 14 generations. Again, if we take the higher number it gives a decrease of 8.25 generations. It seems then, that if two lines of a given clone are subjected to different temperatures, they tend to diverge physiologically more rapidly than when kept at the same temperature.

## Summary of Experiments Involving Different Environments.

As previously shown, when two lines arising from daughter-cells are subjected to similar environmental influences the property of fusing with each other's detached fragments of protoplasm ceases after approximately 21.58 days and 22.25 generations. By keeping one line in 10 per cent. hay infusion at room temperature in front of a window, such intra-clonal differentiation could be greatly hastened by altering (in only one respect) the other, as follows:

| Factor imposed. | Differentiation Exhibited. Days. | Generations. |
|---|---|---|
| 25 per cent.[1] hay infusion used | 8 | 9 |
| 1 per cent. sucrose added | 6 | 7 |
| N/10 saline added (unfavorable) | | |
| 0.01 per cent. acetic acid added (killed organisms) | | |
| 0.025 sodium carbonate added | 7 | 7 |
| 1 per cent. alcohol added (fluctuated between + and −) | | |
| Kept in darkness | 13.25 | 14.5 |
| Kept at 15 degrees C.[2] | 16.5 | 12.25 |

[1] The other line kept in pond water instead of 10 per cent. hay infusion.

[2] The other line kept in darkened place to counteract darkness of refrigerator.

## III. Identical Environments.

When the experiments involving similar environments had been completed, and before the experiments described in section II had been performed, it *seemed* that the inability of an organism to fuse with a protoplasmic fragment which had been severed from a related individual was due to inherited physiological variations. However, it was found, as shown in section II, that by employing different environments the negative state was reached in a much shorter time than was the case when the conditions were unaltered. These results indicate either (1) that the different environments have brought about heritable physiological changes which influence the character of the reactions, or (2) that the different environments have brought about temporary physiological changes which do not persist after the organisms are returned to identical surroundings. In other words: the so-called "similar" environments may have, in reality, been different. As Jennings ('16) pointed out, and Hargitt and Fray ('17) and Phillips ('22) have shown by feeding *Paramecia* on pure cultures of bacteria, food is an important factor in bringing about modifications. There seems to be no doubt that certain forms of bacteria prevail in one concavity while in an adjoining concavity the flora may be entirely different. *The Arcellæ* in hay infusion cultures subsist principally on the bacteria by which they are surrounded; therefore, one should not be surprised if physiological differences are exhibited between organisms kept in different containers.

Several methods might be employed in determining whether or not the bacterial flora available to the organisms of two contrasting lines are similar:

1. By making cultures of the medium in which they are living, and determining the kinds and relative proportions of bacteria present through the use of differential culture media. However, since little is known about hay infusion bacteria, this would be a difficult undertaking.

2. By isolating a pure strain of suitable bacteria and feeding them to the organisms under aseptic conditions.

3. (*a*) By frequently transferring small quantities of the medium from one concavity to the other, and (*b*) by placing both

organisms in the same concavity and removing the new individuals as they appear.

Methods 1 and 2 were not undertaken, but both (*a*) and (*b*) of method 3 were utilized.

Four clones, J, K, Q, and R were started and each of these was divided into two lines upon the first division. Individuals from these lines were transferred to new concavities every other day, and upon each transferral of the organisms a quantity of the culture medium from the old concavity in which one line had been growing was placed in the new concavity in which a specimen from the other line was to be confined, and *vice versa*. Thus, each time an organism was placed into fresh culture medium it brought with it some of the bacteria from the old culture in which it had been living; but in addition to this, bacteria from the old culture in which its sister line had been growing were also introduced, so that any difference in the flora existing in the two old concavities were distributed to the new concavities alike. Tests were made between representatives of the two lines as had been done in former experiments, and it was found that cross-fusion did not cease after twenty or twenty-five generations, but persisted as long as the experiments were continued; or in clone J until the 42d generation, clone K until the 47th, clone Q until the 65th, and clone R until the 58th. After obtaining cross-fusion in clones J and K for 42 and 47 generations respectively, Petri dish cultures were made of the two lines in each clone by putting four individuals in a Petri dish containing 25 c.c. of 10 per cent. hay infusion. These were placed before a window where they remained undisturbed from July 31 until August 25.

Another series of experiments was begun in which attempts were made to bring related specimens, that had been reacting negatively towards each other's fragments, to a state in which cross-fusion would take place between them, by frequently exchanging small quantities of culture medium between the concavities in which they were living. Three variations of this system were employed: (*a*) by cross-transferring a small quantity of culture medium from the old receptacle of one line to the new container of the other each time the organisms were conveyed to fresh culture medium—thus bringing their environments

to a common state; (*b*) by transferring medium only from one line to the other—thus making the conditions in the latter like those in the former; (*c*) the environments of both lines were changed through the use of a different culture medium; these were then brought to a common identity by exchanging small quantities of culture medium between them.

Members from the Petri dish cultures of clones J and K were used for this purpose. When tested on August 25 it was found that the two lines of these clones reacted negatively towards each other's fragments. By estimating the number of individuals in each Petri dish, and knowing the number of days the cultures had been running and their usual rate of reproduction, a fairly accurate determination could be made of the number of generations represented in each line. In every case the number used was slightly under the estimated number. By following this procedure the figure obtained on August 25 were: for clone J, line **j**–66 generations and line **jd**–60; for clone K, line **k**–70 and line **kd**–68. The same day three types of experiments were begun in which both of these clones were used.

(*a*) Two small drops of culture media were interchanged between the old receptacles containing specimens from one line and the new concavities into which a specimen from the other line was to be placed each time the organisms were conveyed to fresh cultures (every two days). At intervals of two or three days observations were made of the reactions between individuals from the two lines. In clone J the interactions of lines **j** and **jd** continued to be of the shattering type for 18 days (until September 12), when cross-fusion was exhibited (line **j**–84 and line **jd**–80). From this time until the experiments were discontinued on November 1 (**j**–124 and **jd**–119), individuals from one line would readily fuse with fragments that had been severed from members of a sister line. In clone K the interactions between lines **k** and **kd** were of the shattering type for 20 days (until September 14). From this time on, cross-fusion between the two lines was exhibited, though several shattering reactions were also observed. The experiment was discontinued on November 1, at which time line **k** had undergone 135, and line **kd** 132 divisions since clone K was started on June 25.

(*b*) In the following experiments attempts were made to bring

lines jd and kd to the conditions of lines j and k respectively, by conveying some of the culture media in which the latter had been living to the concavities containing the former. Under this treatment individuals from lines j and jd reacted negatively towards each other's fragments during the succeeding fifteen generations (until September 9). After this cross-fusions were the usual occurrences, though occasional shattering reactions were encountered. When the experiment was discontinued on November 1 line j represented 129 generations and line jd 120 generations since the beginning of clone J. The reactions between lines k and kd continued to be of the shattering type until September 16 (for 24 generations). From then until the experiment was discontinued on November 1 (line k–135 and line kd–133) most of the interactions were positive.

(*c*) In these experiments specimens from clone J, lines j and jd and clone K, lines k and kd were taken from the Petri dish cultures and placed in concavities containing 10 per cent. hay infusion plus 1 per cent. sucrose; but after remaining in this solution for six days, the organisms had decreased so much in size that they were put back in standard medium. Every other day small quantities of culture media were exchanged between the receptacles in which sister lines were kept. The organisms subjected to 1 per cent. sucrose not only decreased in size, but their physiological states were so altered that they no longer fused with fragments from closely related individuals which had been kept in standard culture medium—see section II, (*b*). Thus, it is evident that an environment different from what existed before had been established.

Upon testing by the usual method, it was found that the shattering type of reactions persisted between specimens of line j and line jd for 18 days and 19 generations, but from then on, cross-fusion was exhibited. The experiment was discontinued on Nov. 2d, at which time line j represented 125 generations and line jd 122. In clone K shattering reactions were observed between line k and line kd until the 23d day and 17th generation after the experiment was started. Later observations yielded a much larger proportion of shattering reactions than were encountered in any of the other experiments, but a number of cross-fusions did take place between the two lines. When the

experiment was stopped on Nov. 1st. line k had undergone 119 divisions and line kd 122.

The results obtained in the above experiments indicate that the changes produced in the protoplasm of related individuals reproducing vegetatively, are at least greatly influenced by the environment—in fact to such an extent that two lines which have been negative to each other's protoplasmic fragments for several weeks can be brought back to a common condition in which cross-fusion will take place between them within approximately 20 days. However convincing this evidence seems, it was considered advisable to obtain further proof. To that end, a series of experiments was planned which would extend the observations over a longer period, and at the same time remove any question as to the identity of the environments in which the two lines of a clone were existing. It is a matter of common knowledge, that when *Arcella* divides the newly formed shell is lighter in color than the old shell, and that this difference in shell coloration continues for some time after division takes place. Therefore an individual possessing a dark shell was taken from each of the two diverging lines of a given clone and placed in the same concavity. Then daily examinations were made, and when two specimens with light-colored shells appeared, they were removed and tested for inter-protoplasmic reactions; while the two dark-shelled organisms were transferred to fresh culture medium. It might be said in this connection, that the morphological differences between specimens from the two lines, were usually also great enough to enable a careful observer to distinguish one from the other.

On November 1, 1922, four experiments of this type were started, involving clone J, line j–124 and line jd–119; clone K, line k–135 and line kd–132; clone Q, line q–65 and line qd–65; and clone R, line r–58 and line rd–58. The two lines of each of these clones had previously been brought to a common condition by exchanging small quantities of culture media from one to the other, as described elsewhere; so that individuals from one line would fuse with fragments of members of the other at the beginning of these experiments. So then, it was to be expected that the immediate progeny of two such organisms would also exhibit the cross-fusion phenomenon.

Individuals from the two lines of these clones were confined in the same concavities for ten days. During this time frequent tests showed cross-fusion in every instance: consequently on November 10 the related lines were separated again, by placing the individuals in different Petri dishes containing 25 cc. of 10 per cent. hay infusion. At intervals of two weeks the number of individuals in the Petri dish cultures were estimated, and the approximate number of generations represented determined. Then fresh Petri dish cultures were made, and thus the lines were perpetuated until January 5, 1923.[1] On January 5 tests were made for cross-fusion between the two lines of these clones, with the result that all of them gave the shattering reaction. Then an individual from line j was put in a concavity with an individual from line jd. In a like manner the two lines of the other clones were brought together. During the next five days the interprotoplasmic reactions were universally negative, while on the sixth day they were all positive. Cross-fusion between the related lines continued until the experiments were stopped on January 15. At that time clone J had been observed for 188 generations, clone K for 200, clone Q for 126, and clone R for 122.

These results present a strong argument in favor of environments as the principal modifying agent; but the skeptic might say that perhaps the two lines were confused while being kept in the same container, and what seemed to be cross-fusion between very distantly related individuals was really positive interactions of very closely related specimens. That such was the case is very improbable; for in the first place, especial care was taken to avoid that error, and secondly, the results obtained were too uniform. The same mistake would hardly have been made in every experiment at the same time. However, the most convincing evidence obtained was made possible by the sudden appearance of a double form in a Petri dish culture of clone Q, line qd. This abnormal specimen was discovered on November 21, 1922, and seems to have appeared because of the failure of the shells to completely separate at the time of fission. It is

[1] An objection might be raised to mass cultures on the grounds that conjugation might take place between some of the organisms, thus disturbing the germinal constituency of the individuals concerned. This possibility was taken into consideration and guarded against, and it can be said with assurance that conjugation did not take place among the specimens used in this investigation.

shown contrasted with a normal individual in Fig. 4. (See Reynolds '23.)

As can readily be seen, the morphological differences between this abnormal specimen and a normal individual were quite marked; consequently they could be kept in the same concavity without any danger of confusion. The abnormal form could be reared, and it was found to breed true to type. By January 12 34 generations had been obtained from the abnormal *Arcella*, while the normal individuals of the line from which it sprung had given rise to 37 generations. During this time, a period of 52 days, the normal and abnormal organisms had been kept in different receptacles, and no exchange of medium between them had been made. In accordance with other findings, therefore, they should have reacted negatively towards each other's fragments of protoplasm—even though both had possessed the same morphological characteristics. In testing this out on January 12 it was found that they exhibited a violent shattering reaction. On the same day an abnormal specimen **Ab**–34 was put in the same concavity with a normal organism **qd**–123, both of which belonged to the same line (**qd**) of clone Q. By making daily tests[1] it was found that the interactions continued to be negative for three days. From the fourth day on, cross-fusion took place between them readily.

Another experiment involving the abnormal form was also started on January 12. In this case a normal individual **j**–185, which was descended from a specimen taken from a wild culture on June 26, 1922,[2] was placed in a concavity with an abnormal organism **Ab**–34, after it had been demonstrated experimentally that one would not fuse with fragments of the other's protoplasm. Then the usual procedure of daily testing the interactions between the progeny of these two organisms was carefully practiced. During the first five days the involved protoplasms were shat-

[1] In this and the following experiment all amputations, and subsequent observations of protoplasmic reactions, were made without removing the organisms from their containers.

[2] While the two organisms used in this experiment belonged to the same species, and probably at some time in the past had a common ancestor (since their progenitors were isolated from the same wild culture); their relationship was certainly remote, for the two clones to which they belonged had been under observation for nearly seven months.

tered to pieces upon making contact, after which the animal would move off, leaving the remnants of its own and the fragment's protoplasm behind. On the 6th, 7th, and 8th days contact was followed by a slight shattering of protoplasm, and later some or all of the remnants were appropriated by the reacting organism. On the 9th, 10th, 11th, and 12th days of coexistence one organism would fuse with a fragment of the other practically, if not quite, as readily as with one of its own fragments. No further observations were made after the twelfth day.

### Summary of Experiments Involving Identical Environments.

By continually interchanging small quantities of media from old cultures of one line of a given clone to new cultures of the other line, cross-fusion between the two lines can be prolonged for sixty-five generations (perhaps indefinitely?). After two lines of a clone have ceased to fuse with each other's fragments, the property of cross-fusion can be restored by: (*a*) frequently exchanging small quantities of media between the cultures—thus bringing them to a common state; (*b*) frequently transferring small quantities of medium from only one culture to the other—thus bringing one line to the condition of the other; (*c*) creating a different environment in both lines by adding 1 per cent. sucrose to the culture medium and then exchanging media between them frequently. Quicker and more satisfactory results can be obtained, if members of the two lines to be tested are cultivated in the same container. By this method, two lines of a clone that have been negative to each other's protoplasm for months, can be brought back to a state of cross-fusibility—even though they are removed from the clonal parent by as many as 200 generations. A normal specimen was put in the same concavity with an abnormal relative. Though reacting negatively in the beginning, they readily fused with each other's fragments of protoplasm after living together for four days. An unrelated (?) normal specimen was placed in the same container with an abnormal *Arcella*. In this case the shattering reaction was exhibited until the ninth day of coexistence; from then on, fusion occurred readily between one organism and pseudopodial fragments of the other.

## Discussion.

Before entering into a discussion of the results obtained in these investigations, it is perhaps advisable to show that the method employed is sound, and that it offers some advantages over the methods which have been utilized by others. As stated in the introduction, this is the first time that the interactions of protoplasmic masses have been used in studying the subject of Heredity and Environment. It was not until the recent appearance of the first article of this series that anything was published concerning the fact that certain of the shelled rhizopods are capable of reappropriating, by fusion, fragments of protoplasm which have recently been detached from their cell-bodies. In this paper it is clearly pointed out that "the severed fragments are not recovered as food, but enter again immediately into the protoplasmic structure of the cell-body." Later work has strengthened the evidence for such a contention. In fact, the findings in these experiments with *A. polypora* substantiate all of the conclusions drawn from the work on *Difflugia*, except paragraphs 3 and 5 of the general summary.

In regard to paragraph 3: In *Arcella*, fusion has been observed to occur at the ends of pseudopods, as well as along an extended mid-region. This difference in behavior of the two genera may be due to the difference in the nature of their pseudopodial formation. As to paragraph 5: In six cases out of approximately 1,000 two enucleated fragments have been observed to fuse with each other; though in such instances they were severed from the same cell-body at the same time, and were almost in contact in the beginning.

In addition to the data on protoplasmic fusion given in Article No. 1: the "shattering" reaction is described in the introduction of the present paper. This is the kind of reaction usually observed when an organism made contact with a fragment severed from a distantly related individual. It was found that in such cases there was a mutual attraction between the organism and the fragment—even though their protoplasms were shattered upon making contact. If the fragment came from a closely related specimen fusion occurred. When a fragment of protoplasm was severed from a different genus (*Difflugia*) and placed

near an *Arcella* the two bodies did not attract eachother. This was done on ten different occasions; each time the fragment was placed within 100 micra of the organism, but neither body showed any response to the other's presence. Chance wanderings should have brought the two masses together in a certain percentage of cases. This did happen on two occasions out of the ten tried, but nothing resulted from the contact. In both instances the animal moved on as if the fragment had been merely a mechanical obstruction.

Another observation which is probably worthy of mention in this connection, is that under ordinary circumstances two *Arcellæ*, which would react negatively towards each other's fragments, may come in contact with each other's pseudopods without causing any disturbance; but when a protoplasmic fragment of one of these organisms is in their midst, it was frequently observed that their pseudopods would be shattered upon making contact.

With these various facts established, it seems evident that the inability of an organism to fuse with a pseudopodial fragment is due to a difference in the physiological constitution of the protoplasm involved. It is generally considered that morphological differences are merely physical expressions of physiological changes—a kind of cause and effect relationship. If this be true, then physiological tests offer finer distinctions between minute variations than can be perceived in the structure of organisms. Furthermore, as shown by the experiments in which one line of a clone was placed in a culture medium containing 1 per cent. sucrose, physiological changes may persist for a long time after morphological differences have disappeared.

Most investigators in the past have, of necessity, studied variations among protozoa by making use of structural differences. While these are undoubtedly of great importance, they do not offer as delicate a method of determining variations, or as sure a means of detecting them. Anyone who has observed a "shattering" reaction can but be impressed with the fact that a marked difference exists between the reacting masses—a few generations back they were parts of the same individual; they have diverged by means of binary fission until they not only fail to unite when brought together, but are shattered into many pieces. Why does this happen? We can postulate many possible

explanations. It seems most reasonable to think of it as being due to a difference in molecular structure, or ionization; at least the sudden violent shattering is suggestive of an electrical phenomenon. The inability of one organism to fuse with a protoplasmic fragment of another, does not imply that the two organisms concerned differ structurally, nor does cross-fusion indicate that no morphological differences exist. In either case the animals involved may or may not be identical in size, structure and rate of fission.

Maupas ('88, '89), in studying the effect of temperature on the fission rate of infusoria, arrived at the conclusion that under given conditions the fission rate remains the same, and that inherited variations do not exist. Regardless of the important bearing this statement has on the theory of evolution, there was but very little interest exhibited until some time after the publication of Johannsen's ('03) results, in which he enunciated his concept of genotypes. Since then workers in various fields have investigated the subject of Heredity and Environment. Like Maupas, most of them have obtained evidence which seems to emphasize the "fixity" of species. Among these might be mentioned: Johannsen ('09, '11) working with beans, Hanel ('08) and Lashley ('15) with *Hydra*, Jennings ('08, '09, '10) with infusoria, Ackert ('16) with *Paramecium*, and Jollos ('21) with infusoria. However, recently Barber ('07) working with bacteria, Jennings ('16) with *Difflugia*, Hegner ('19) with *Arcella*, Root ('18) with *Centropyxis*, Middleton ('15, '18) with *Stylonychia*, Erdmann ('20) with *Paramecium*, and others, have apparently been able to demonstrate that variations do take place among organisms reproducing asexually—and as Erdmann ('20) says: "The rigid conception of the genotype does not hold true for protozoa."

To enter a field filled with so much conflicting evidence, armed with a new method, was an especially attractive undertaking. The experiments were planned with the idea of duplicating, so far as possible, the various conditions to which protozoa have been subjected by others. The only new factors employed were the method of determining variations which might arise, and a little different way of preparing the culture medium.

The results obtained seem to combine the two diverse opin-

ions, and thus answer some of the hitherto perplexing questions. In the first place, they show that both physiological and morphological variations do appear among protozoa reproducing vegetatively, and that such variations can be hastened by altering the environments; and secondly, that these changes are not due to inherited variations which persist for very long periods of time, for they could be reversed within a few days. Unquestionably, certain heritable variations often arise in cultures, and seem to persist for some time afterwards; but, in our experiments at least, if the organisms in which these changes are manifested are left in the same surroundings, undisturbed, they tend to revert to the normal type, while if placed in a different environment where selection would be affective, or if artificial selection is practiced, the aberrant characteristics may be continued, or even emphasized.

In a similar manner the physiological status of organisms is subject to modifications. Adjustments are constantly being made as a result of the ever changing surroundings. The question seems to resolve itself into one of environmental factors, of which food is by no means the least important. The only difficult factor to control in these experiments was the bacterial growth in the culture media. In fact, the rapid differentiation observed when unlike culture media were used, was probably due to a change in the character of the bacterial flora produced by the action of the environment; for if the action had been direct, in some cases at least, physiological differences should have occurred within a few hours after the organisms were exposed to it.

## Summary and Conclusions.

1. Like *Difflugia*, *Arcella polypora* will, under favorable conditions, reappropriate its detached fragments of protoplasm by fusion.

2. *A. polypora* is not attracted by a pseudopodial fragment of *Difflugia*. If accidental contact is made the fragment is treated as a mechanical obstruction.

3. Fusion will take place between one individual and a protoplasmic fragment of a closely related specimen.

4. Two distantly related individuals, which have not been kept in the same receptacle, will be attracted by each other's

severed pseudopods, but upon making contact the involved protoplasms will be shattered into bead-like masses.

5. When two lines of a clone are kept under similar environments, cross-fusions between them cease after approximately 22 days; while if the two diverging lines are subjected to different environments, the time required for cross-fusions to cease is greatly shortened (varying from 6 to 16.5 days depending on the factor employed).

6. When small quantities of culture media are frequently exchanged between the concavities in which members of two diverging lines are confined, cross-fusions between them apparently continue indefinitely

7. After two related specimens have become negative to each other's protoplasm, cross-fusions between their progeny may be induced by (*a*) exchanging small quantities of culture media between the receptacles in which they are kept (time required was 20 days), or (*b*) by placing both of them in the same concavity (time required was 6 days).

8. Morphological differences are not necessarily associated with different physiological states, as evidenced by the fact that abnormal specimens could be induced to fuse with fragments of normal individuals—and *vice versa*.

9. Physiological changes do occur among the descendants of a single *A. polypora* reproducing vegetatively, but such differences are probably due to environmental influences rather than to hereditary variations.

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## Explanation of Plate I.

Fig. 1. Camera lucida sketches illustrating shattering reaction. *A*, *Arcella polypora* approaching a fragment from another individual of the same species, fragment shown below. × *170*. *B*, The same bodies just after making contact. *C*, A portion of *B* enlarged.

Fig. 2. Diagram of vertical section illustrating shape of shell and position of pseudopods.

Fig. 4. An abnormal *Arcella* which appeared suddenly in a hay infusion culture of clone Q, line **qd**. *A*, Lateral view; *B*, Ventral aspect, showing ellipsoidal mouth; *C*, normal individual of line from which the abnormal specimen was obtained. × *170—camera lucida*.

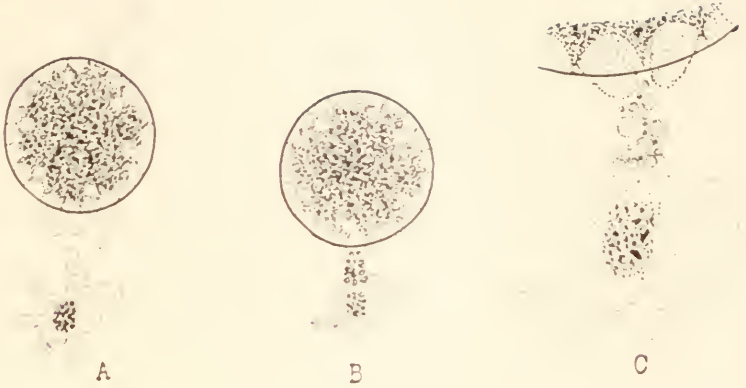


Fig. 1.

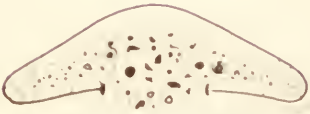

Fig. 2.

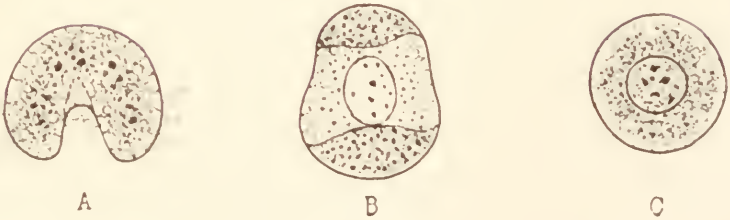


Fig. 4.

BRUCE D. REYNOLDS.

# THE ATTACHMENT OF OYSTER LARVÆ.

THURLOW C. NELSON.[1]

Knowledge of the life history and ecology of the American oyster, *Ostrea virginica* Gmelin, is more extensive than in the case of any other species of lamellibranch. The researches of numerous investigators, notably of Brooks, J. Nelson, and Stafford, give us an almost unbroken history of this mollusc from the egg to the adult. As in most marine bivalves, the eggs of the oyster are shed into the water where fertilization occurs. Subsequent development is rapid, the larva forming a bivalve shell within two days or less. Then there follows a pelagic period of approximately two weeks during which the larva swims about with the aid of the velum, and increases considerably in size. At the close of the free-swimming period the larva settles upon some solid object and remains firmly attached at that point for the remainder of its life. Up to the time of attachment the history of most other marine lamellibranchs is similar to that of the oyster, but once the free-swimming period is ended we find a variety of behavior; the larvæ of *Pecten*, *Modiolus*, and *Mytilus* attach to seaweeds or to other objects, the larvæ of the Teredinidæ and of the Pholads bore within a solid substratum, while those of *Venus* and *Mya* burrow into the bottom.

Concerning the actual attachment of the oyster larva at the close of its pelagic life we have little information, the nature of the process having been deduced in the main from a study of the stages just preceding and just following fixation. Ryder, '82, Huxley, '83, Jackson, '90 and others, including the writer (Nelson, '21 A), believed that the secreting border of the mantle was used to cement the larva fast to the substratum. Stafford, '13, on the other hand, found in his youngest oyster "spat" that the left valve bore a layer about five times as thick as the shell itself, composed apparently of a different material. This thick layer

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covers more than one half of the outer surface of the left valve, extending from close to the anterior ventral edge almost to the umbo, but falling short posteriorly. From his study of the byssus gland in the base of the foot of the larval oyster, Stafford concluded that this organ must furnish the substance used to attach the larva, since the large amount of this material and its position far under the left valve preclude the use of the mantle for this purpose.

It has been my good fortune to observe the actual attachment of oyster larvæ under semi-natural conditions, and thus to be able to fill the gap in the information about this process. Large numbers of full grown oyster larvae were found at 8 P.M., July 23, in the water surrounding our laboratory houseboat on Barnegat Bay. A glass plate measuring 3 x 4 inches was lowered vertically into the water and removed half an hour later, and immediately suspended horizontally in a dish of sea water under the binocular.[1]

Six oyster larvae were moving about over the glass, holding on by means of the very active and highly adhesive ciliated foot. Some were on the upper, others on the lower side of the glass, thus permitting observation of the attachment process both from above and from below. With the velum withdrawn, and slowly

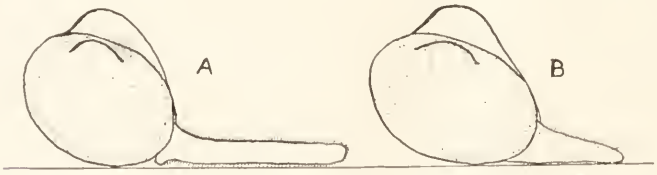


FIG. 1. Illustration of the method of creeping of the oyster larva over a glass surface. *A*, larva with foot fully extended; *B*, larva with foot contracted and body pulled forward. Larvæ shown from the right side.

rolling the shell from side to side, the larvæ extended the foot to a distance of about 0.25 mm., attached the distal end and then a contraction of the foot pulled the body forward. The "heel" of the foot next being attached, the body was swung part way around; the tip of the foot was again extended in the new direction and the process repeated (Fig. 1). When first observed at

[1] Jackson, '88, J. Nelson, '08, and Stafford, '10, used this method of obtaining oyster spat; but no one of these authors records having witnessed the actual attachment of the larva.

8.35 P.M. the larvæ were describing circles a little over 1 mm. in diameter. As they continued moving around the radius of the circles was gradually diminished, until at 8.46 the first larva came to rest With the foot extended to about one half its full length, its distal end flattened in contact with the glass, the larva swung the body until the foot occupied the median position, with the left valve against the glass and inclined to it at about 30 degrees (Fig. 2). In this position the ventral edge of the left valve almost touches the substratum. The ventral border of the mantle was extended until it came in contact with the glass where it remained for 2 minutes, and then was withdrawn. The foot, with its cilia beating very feebly, was then slowly drawn in, and 11 minutes after the larva ceased circling the foot had entirely disappeared between the valves, and fixation was accomplished. The method of attachment of the larvæ on the upper and on the lower side of the glass was the same; it illustrates, in the case of those larvæ attaching from below, the power of the foot in holding the left valve firmly against the substratum while fixation is being effected.

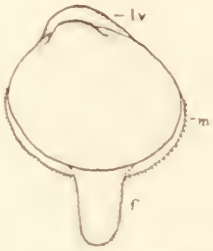


FIG. 2. The position taken by an oyster larva during fixation, shown from right side. *f*, foot; *l. v.*, umbo of left valve; *m*, mantle.

The great importance of the foot to the full-grown oyster larva was recognized by Stafford '10, who described its creeping movements. The manner in which the larva cements itself fast was deduced by him ('13, p. 65) solely from histological evidence. He concluded that the mantle could not furnish material in sufficient quantity nor rapidly enough to be of use as the organ of fixation. As he says: "The usefulness of the foot as an organ of locomotion, as a clinging organ, as an organ of fixation, had appealed to me for some time but I had no direct evidence to support the view that it was really the organ of final attachment."

Stafford found that a large part of the base of the foot of a full-grown oyster larva is occupied by the byssus gland which consists of three lobes communicating with a duct which opens

to the outside at the heel of the foot. The cells of this gland are gorged with a transparent secretion which is not found in the much shrunken gland which characterizes the larva immediately after attachment. He believed that fixation was accomplished by the larva extending the foot until the heel came to a position near the upper anterior edge of the valve, and that the byssus gland then discharged its secretion, which, flowing between the left valve and the substratum, soon hardened and held the larva fast. Stafford believed that the mantle played no part in the fixation process.

My own observations show that Stafford was right in considering the foot as the organ of final attachment. It is not thrust forward, however, as he held, but is brought to the median position. The extrusion of the mantle for a short period evidently aids in the quick and economical distribution of the cementing fluid as it is poured out of the byssus gland at the ventral edge of the left valve. This secretion hardens in less than 10 minutes. In Fig. 3 are shown a number of newly attached larvæ

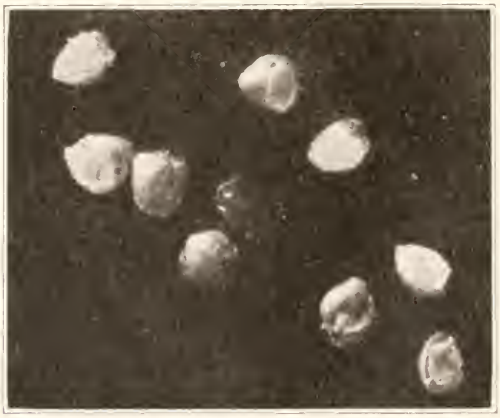

FIG. 3. Oyster larvæ removed just after attachment and photographed from the left side to show the area covered by the cementing substance from the byssus gland. Magnified about 40 diameters.

photographed from the left side to show the area covered by, and the distribution of, the cementing material.

The use of the foot in creeping over surfaces has been described in a number of larval bivalves, and it is the chief means by which the young molluscs obtain foothold in a favorable locality.

Kellogg, '99, showed the importance of the foot in aiding the larva of *Mya* to obtain attachment. Sigerfoos, '07, noted that larval shipworms possess a powerful foot by means of which they creep rapidly over surfaces and select spots favorable for fixation to the exclusion of those which are not suitable. Field, '09, states that the larvæ of *Mytilus* creep from unfavorable situations upon seaweeds to locations which are better adapted to them. Belding, '10, describes the foot of the larval *Pecten*, a most important organ, since it is used for swimming as well as in crawling. Belding, '12, showed that in the full-grown larva of *Venus* the foot likewise is used in swimming as well as for crawling, and for procuring attachment.

That the use of the foot enables oyster larvæ to exercise some selection in seeking a place of attachment is evident from the wide differences in the number of spat procured when various kinds of cultch are placed together in the water. I have recently shown (Nelson, '23,) that oyster larvæ will not attach to shells which are extensively pitted by the boring sponge, *Clione*, or which are badly corroded and which present surfaces that are microscopically rough.

For some hours before oyster larvæ set they may be observed moving about over various surfaces with the velum extended, its cilia beating actively; and with the foot protruded and bent at right angles, thus bringing the distal half in contact with the substratum. The foot remains quiescent in this position while the larva slowly "skates" along, as it were, propelled by the velum, but aided somewhat by the cilia on the foot. Then, swimming upwards, the larva progresses a short distance through the water and drops down to the substratum to continue as before. In this manner the larva may wander over an appreciable area before it eventually attaches (Nelson, '21–A).

In this connection it is well to correct the idea held by oyster growers and by some scientists also, that attachment of the oyster larva occurs when its shell becomes so heavy that the animal sinks to the bottom, unable longer to swim. For example, Stafford ('13, 34) says: "when presumably the larvæ become too heavy to swim with ease, settle towards the bottom, creep about, and select some clean solid surface upon which they fix themselves."

That such is not the case must be evident to anyone who has observed extensive oyster sets on the bottoms of boats, or upon the bottoms of floats kept at the very surface of the water (Nelson, '17,). The full-grown oyster larva, so far from being helpless, is capable of more powerful swimming than at any time during its pelagic life. During the course of the development and growth of the larva the increase in size of the velum is more than commensurate with the increase in the weight of the shell. Plankton samples taken during the calm of early morning show large numbers of full-grown oyster larvæ swimming at the surface. At times the larva may project the foot into the surface film and, withdrawing the velum, may hang suspended by the foot and slowly rock the shell from side to side, much as in the familiar habit of pond snails hanging from the surface film.

Full-grown oyster larvæ are found mostly at the bottom because they develop during the last two days of their pelagic life a strong positive stereotropism, and since fixed objects are most abundant on the bottom the larvæ mainly congregate there. The intensity of attachment which occurs at the very surface of the water when suitable cultch is available, precludes the idea that increased weight of the shell or even positive geotropism are factors exclusively governing the time of fixation.

The use of the foot by oyster larvæ in crawling over surfaces is similar to that reported by Crozier, '21, who found that the adult *Lima* oriented itself away from the light by attaching the tip of the foot to the substratum after bending the free portion away from the source of illumination. The creeping of oyster larvae is also strikingly similar to that observed in the fresh-water Sphæridæ, and especially in the young of *Corneocyclas* when first liberated from the brood pouches of the parent (Nelson, '21–B).

In sessile forms, such as the oyster, which remain fixed on the spot where the larvæ set, keen competition follows when large numbers settle at one time. During heavy sets 1,000 larvæ may attach to one oyster shell 8 cm. long, upon an area which will permit not more than 6 oysters to grow to breeding age. It is significant that at such times of abundant settlement, when the water over the oyster beds may show as high as 250 full-grown larvæ per liter, the frequency of attachment per unit area of available cultch does not exceed a certain maximum.

Fig. 4 shows in a circle 6 mm. in diameter a group of 40 oyster larvæ just attached to an experimental shell. Unfortunately I have never witnessed the behavior of two oyster larvæ coming into contact with each other while circling about over a surface, but an examination of shells taken at times of maximum settlement indicates that the movements of the larvæ prior to final fixation not only aid them in finding a spot suitable for attachment, but that this behavior also tends to keep the larvæ separated from one another. The proximity of a neighboring larva could presumably be recognized only through touch, hence it is conceivable that here and there individuals failing to come in contact while circling about might attach close to one another, as occurred in six instances in Fig. 4. Examination of the most

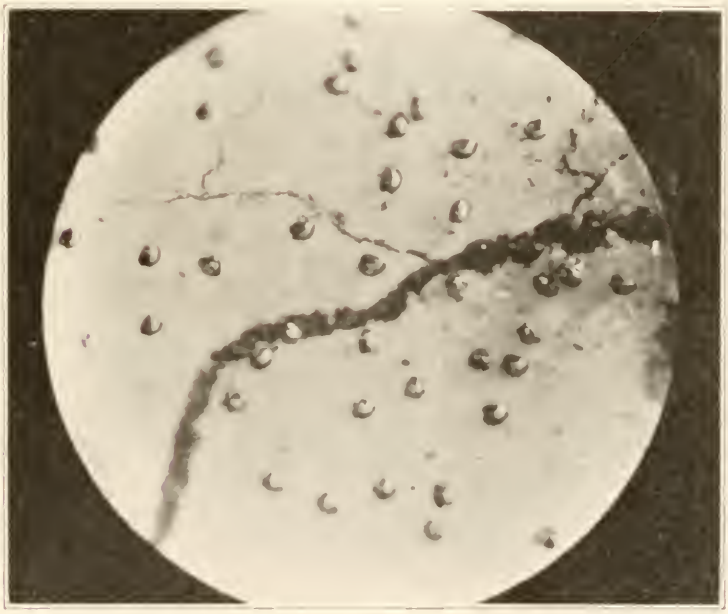

FIG. 4. Newly attached oyster larvæ on the surface of an oyster shell. Within this circle, 6 mm. in diameter, are 40 larvæ. Magnified about 15 diameters.

heavily set shells in our collection shows out of 500 newly attached larvæ only 57, or 11.4 per cent., which are within .5 mm. of another larva. This distance is taken since it represents the approximate radius of the circles described by the larvæ when first observed. Studies of the behavior of other bivalve larvæ

during the attachment period should give interesting information as to the prevalence of this type of behavior.

## Summary.

Full-grown larvæ of the oyster shortly before attachment move over an appreciable area of solid surface testing it out with the foot.

Actual attachment is preceded by circling movements, the larva finally coming to rest with the left valve held in contact with the substratum by the foot.

The suggestion of Stafford, 13, that the foot is the organ of attachment has been confirmed by direct observation. The use of the foot and of the mantle during fixation is here described.

The circling movements of the larva while crawling over a substratum not only aid it in obtaining a favorable foothold, but probably are also instrumental in producing a fairly even distribution of the spat.

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# BIOLOGICAL BULLETIN

## THE BEHAVIOR OF THE NUCLEUS AND CHROMOSOMES DURING SPERMATOGENESIS IN THE ROBBER FLY *LASIOPOGON BIVITTATUS*.[1]

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### INTRODUCTION.

During the growth period of the primary spermatocytes in *Lasiopogon bivattatus* Lw.,[2] nuclear changes occur which transform the spherical nucleus into a series of ramifying chambers enclosing the chromosome threads. Conditions in these nuclei suggest that the stainable threads are axes or cores of considerably larger bodies, which are mainly hyaline. The present account is concerned primarily with these features, although it includes a description of spermatogenesis up to the second spermatocyte division.

The observations are based on two specimens, collected near Sheridan, Wyoming. Both were fixed in strong Flemming and stained with iron hæmatoxylin. One (2055) was counterstained with light green. The technique employed was the same as that regularly used in preparing dipteran material, and the fixation is good in both specimens. So far as we know the peculiar nuclear behavior is characteristic of the species and does not represent an accidental abnormality.[3]

[1] A preliminary account of this was given before the Society for Experimental Biology and Medicine and published in the Society's Proceedings (see Metz and Nonidez, '22).

[2] We are indebted to Mr. Jas. R. Hine for the species determination.

[3] This is supported by the fact that conditions somewhat similar have been found in two other species of flies (discussed below). In these our material is sufficiently extensive to prove that the conditions are normal.

The testes of *Lasiopogon* resemble those of *Asilus* and many other members of the Asilidæ, in being a pair of long, coiled tubes, with the spermatogonia near the apex, and successive stages in spermatocyte development progressing in serial order toward the base. This serial orientation of stages makes it relatively easy to follow the succession of events during spermatogenesis.

## Spermatogonia.

At the extreme tip of the testis lies a small mass of disorganized, nutritive material containing only a few cells. Adjacent to this lie the spermatogonia, many of which are connected with the nutritive mass by long protoplasmic processes. This condition differs from that in many other asilids. In the latter the nutritive mass lies in the center of the tube, a short distance from the end, and is surrounded on all sides by spermatogonia.

The spermatogonia present no unusual features except in late prophase. There the nuclei appear to undergo a change similar to that observed in the primary spermatocytes. The nuclear membrane breaks down or becomes irregular in outline and the chromosomes appear to lie free in the cytoplasm, each surrounded by a hyaline zone of about the thickness of the chromosome. The other stages in spermatogonial development appear to be essentially the same as those in *Asilus sericeus* (Metz and Nonidez, '21).

The spermatogonial chromosome group probably consists of six pairs, or possibly of five pairs of autosomes and an unpaired X-chromosome. Fig. 1 only shows five pairs, but the nucleus may not be entire. One or two other figures (not suitable for drawing) seem to include six pairs, and spermatocyte metaphases almost certainly include six chromosomes (*e.g.*, Fig. 12).

## The Growth Period.

Owing to the scarcity of final spermatogonial division figures and to the fact that the final spermatogonia are not readily identified by size differences, as they are in some asilids, it is difficult to detect the boundary between spermatogonia and spermatocytes. However, the earliest growth stages seem to repeat the spermatogonial prophase changes, as in the case of *Asilus sericeus* (*l. c.*, p. 171); and so far as we can determine they show essen-

tially the same features as does *Asilus*. Hence they will be passed over rapidly.

The pairs of chromosomes appear soon after the final spermatogonial telophase as granular aggregates (Fig. 2). They are less dense and clear cut than in *Asilus*, and more nearly resemble those in *Dasyllis* (Metz, '22). These aggregates soon loosen up, overlap, and spin out into long, granulated threads, intermingled in a net-like structure. The nucleolus, a dense, spherical body, is prominent from the earliest observed stages (see special account of the nucleolus below).

Soon after the growth period has begun, the chromatin threads become drawn out so finely that they cannot be traced far, individually. They are heavily granulated, with prominent chromomeres. From their history and from their subsequent behavior, as well as from analogy with other forms, they are assumed to be bivalent, although no duality is visible in their structure. There is no conspicuous polarization of the threads at this time, nor is there any evidence of a synizesis or dense contraction stage. The nucleus is clear cut in outline and is of the usual more or less spherical shape, as is also the nucleolus.

At this point changes set in which accompany one another as growth progresses. The nucleolus elongates and eventually becomes double; the chromatin threads undergo a gradual condensation; and the smooth contour of the nucleus becomes broken by a series of invaginations and evaginations, at first more or less irregular, but soon conforming to the outline of the thread-like chromosomes. It is difficult to determine what physical changes occur in the cell; but the nucleus acts as if its tension were released, so that the nuclear membrane offered no resistance to movements of the cytoplasm or of the chromosomes. The former appears to flow in around the chromatin threads, while the latter become extended out into the cytoplasm. Soon the nucleus is converted into a series of long lobes or pockets ramifying throughout the cell. Each pocket, as a rule, encloses only one (bivalent) chromatin thread. These changes are represented by the cross sections shown in Figs. 2 to 4. The surface of the nucleus is tremendously increased by this process, but the volume is so difficult to estimate that we are unable to determine whether it is affected or not.

Little or no regularity of plan is to be seen in the configuration of the nucleus. Its shape is varied and often bizarre. Apparently the lobes ramify at random, following the contour of the long, more or less twisted threads. The nucleolus usually lies in a lobe or pocket by itself, or in a chamber at the junction of two or more lobes.

The lobes themselves are of various shapes, flat, pocket-like sheath-like, cylindrical, or of intermediate sorts. It is to be noted, however, that all of the nuclei undergo the same series of changes and show essentially the same features. There is no irregularity in this respect.

The maximum of distention and distortion of the nucleus is reached soon after the process begins, and while the chromatin threads are long and slender. At this time the lobes are so attenuated and slender that the nucleus is almost lost to view. For the most part it can only be seen as narrow, winding channels, ramifying through the cytoplasm. These channels often extend almost or quite to the periphery of the cell. Since each channel usually contains a single (bivalent) thread, the threads thus become almost completely isolated from one another.

Subsequently the threads undergo a gradual condensation, and the lobes a coincident contraction, as growth progresses (Figs. 5–9).

When the detailed structure of the lobes is examined, at any stage, it is seen that almost invariably the deeply staining chromatin thread (chromosome), extends through the center of the hyaline lobe like a core, if the lobe is cylindrical, or lies midway between the sides, if the lobe is flat. It is separated from the membrane by a transparent layer of approximately uniform thickness. The structure might be likened to an insulated wire, in which the chromosome represents the wire and the hyaline region the insulation.

As the lobe is followed beyond the chromosome it is seen to become narrow or closed. The maximum thickness is almost always in the region occupied by the chromosome. This suggests that the chromosome is surrounded by a cortex of dense or gelatinous material which holds off the nuclear membrane.

No essential change in these conditions is to be observed as the chromosomes condense and the lobes contract. The two

processes apparently go together. As the chromosome thickens, the surrounding layer thickens, increasing the diameter of the lobe in that region. This process gradually progresses up to the prophase, when, as the chromosomes approach their maximum condensation the lobes become decreased in size. In the late growth period there is a suggestion of a secondary lengthening out of the chromosomes and the lobes into a more ramifying condition such as exists at an earlier stage, but the change is not extreme, and may not be constant. It suggests, however, the stage in *Asilus sericeus* (*l. c.*) in which the threads spread apart after undergoing contraction. This also occurs relatively late in the growth period.

In *Lasiopogon* there is no "contraction stage," such as occurs in *Asilus;* but throughout most of the growth period there are some indications of a polarization of the threads toward the nucleolus; and often faint connections are to be seen between the two (Figs. 7 and 8).

During the prophase, and up to a late stage in metaphase, the hyaline layer around the chromosomes remains conspicuous, as shown in Figs. 10–13. The spindle fiber may often be traced to a projection of this layer corresponding to that of the chromosome, and thence through the layer to the chromosome.

Not enough anaphase figures are present in our material to permit us to follow the chromosomes through this stage.

During the late growth period and the first spermatocyte division the mitochondria become very prominent, and by darkening the cytoplasm, aid in bringing the outline of the chromosomal sheath into relief. In metaphase large mitochondrial rods are frequently present which are almost as dense as the chromosomes. These are seen longitudinally in Figs. 10 and 13, and in transverse section in Figs. 11 and 12. It is to be noted that they are not surrounded by a hyaline cortex comparable to that around the chromosomes.

### The Nucleolus.

The nucleolar behavior during the growth period is somewhat different in our two specimens. In one (2056) there is a single nucleolus in the early growth stages,while in the other (2055) there are two. In the following description the two specimens are considered separately.

In number 2056 the nucleolus is prominent from the beginning of the growth period. It grows gradually and serves as an additional criterion for seriating the growth stages. When the nucleus begins to become irregular in outline, the nucleolus elongates and becomes bilobed, then double. One portion is large, dense and irregularly shaped; the other is smaller, less dense and nearly spherical—resembling chromatin in texture. The two parts are often connected by a dark, thin thread. In the late growth stages the large, irregular portion frequently lies near the periphery of the cell, suggesting that it may be cast off. We have not been able to determine its fate, on account of the scarcity of prophase figures, but it seems to disappear before the chromosomes go on the spindle. The smaller portion appears on the spindle in its characteristic spherical form, but reduced in size, and presumably represents the sex chromosomes.

In specimen 2055 there are normally two, equal sized, spherical nucleoli in the early growth stages. Just before the nucleus begins to become lobulated the two nucleoli come close together and apparently unite, although it is possible that one may degenerate after they become approximated. Following this the nucleolus becomes bilobed as in specimen 2056, and subsequently its history is the same as that of the latter.

In both specimens the nucleolar structures are surrounded by a hyaline cortical region similar to that of the autosomes, although it may be noted that the cortex of the large, irregular portion is usually thinner than that of the small, spherical part which probably represents the sex chromosomes.

## Discussion.

The constancy of the hyaline zone around the chromatic threads in the nuclear lobes we interpret as indicating the presence of a relatively solid (gelatinous) cortical layer enveloping the respective chromosomes. The presence of the layer is revealed, of course, by the structure of the lobes. If the nuclei were spherical it would not be detected. This raises the question as to whether the condition is restricted to the present species, or is of more general occurrence but usually invisible.

We have noted above that evidence of the hyaline zone is to be seen in spermatogonia as well as in spermatocytes of *Lasiopogon*.

In the other Diptera known to us,[1] conditions are not usually such as to reveal the presence of a chromosomal envelope if one exists. But indications of one are to be seen here and there, especially in late prophases or metaphases. And in one genus, owing to the unusual nuclear behavior—somewhat like that in *Lasiopogon*—the evidence is strong. This genus is *Ptecticus*, a member of the Stratiomyidæ. It will be considered more fully in another paper, but we may note here that in *P. trivittatus* and *P. sackenii*, the two species we have studied, there is a distinct hyaline zone around the chromosomes of the prophase and metaphase of the spermatocyte divisions, and there are indications of such a structure extending back into the growth period. These features are shown in part by Figs. 14 and 15, from primary spermatocytes of *P. trivittatus*.

This condition, in a family distinct from that to which *Lasiopogon* belongs, suggests that we are dealing with something which is widespread—at least among the Diptera.

In other organisms the nearest approach to conditions like those described above, so far as we are aware, is found in the Orthoptera. Professor McClung informs us that in this group prophase and metaphase chromosomes (of primary spermatocytes) are frequently bordered by clear spaces, differentiated from the surrounding cytoplasm, and that the condition is sufficiently characteristic to suggest the constant presence of a hyaline zone around the chromosomes in these stages. As examples may be cited McClung's published photographs of the first spermatocyte chromosomes of *Mermeria* (McClung, '14, Figs. 129–132) and of *Hesperotettix* (McClung '17, plate 8, Fig. *c*). In all of these the hyaline zone is visible around the chromosomes.[2] We have observed this condition in first spermatocyte anaphases of *Rhomaleum* and find that the hyaline layer agrees in appearance with that in *Lasiopogon*, although we have not as yet tried to trace its history.

The presence of this condition in the Orthoptera and Diptera has prompted us to make a brief comparison with other structures of a somewhat similar nature.

[1] No close relatives of *Lasiopogon* have been examined as yet.

[2] It is probable that other cases of this sort have escaped our notice, although we have examined numerous published photographs of chromosomes with this point in mind.

These include, for the most part, the "chromosomal vesicles" of Conklin ('01, '02, etc.), Wenrich (16), Richards (17) and others. A resumé of the observations on these structures up to 1917 has been given by Richards (*l. c.*), who cites examples in various organisms from molluscs to vertebrates (fish eggs). There is considerable difference in the structure of the vesicles in different forms, but at certain stages in several, if not all, of them it consists of a hyaline outer layer surrounding a more or less definite chromatic axis, suggestive of the lobes in *Lasiopogon*. This is clearly the case in spermatogonial telophases of the Orthoptera and in *Fundulus* eggs (cf. Wenrich, Richards). The stages in which such a condition exists (prophases in the case of *Fundulus*) may be preceded by others in which the chromatic material is scattered through the vesicle, or even largely applied to the surface (*Crepidula*, Conklin '02). In *Fundulus* eggs and in the Orthoptera the anaphase chromosome swells into a vesicle in which the chromatin appears as a granulated network. This persists through the resting stage and then the network condenses into a chromatic thread or rod, extending more or less axially through the vesicle.

If we assume that the hyaline portion of the vesicle is more dense or gelatinous than the surrounding cytoplasm, as is suggested by the appearance and behavior of the vesicles, then the structure bears a close resemblance to that in *Lasiopogon*. On this basis we might conceive of the structures in *Lasiopogon* as arising in a similar fashion—*i.e.*, by the swelling of telophase chromosomes into vesicles, with a diffusion or dispersal of the chromatin (as seen in the early growth stages) and then its recondensation within the vesicle. The term "vesicle" is perhaps inexact in the case of *Lasiopogon* for no limiting membrane, except the nuclear membrane, is visible around the hyaline layer. But the periphery of the latter would, on this view, be considered as analogous to the periphery of the vesicle, whether enveloped in a membrane or not.

The only other description of a structure similar to these, so far as we know, is that of Lee's "chromosomal sheath."[1] In the case of the Orthoptera cited by Lee his sheath evidently corre-

[1] We are indebted to Professor L. W. Sharp for calling our attention to Lee's paper and to de Litardière's criticism of it mentioned below.

sponds to the hyaline portion of the vesicle considered above, and as such is similar to that in *Lasiopogon*. But his conception of a sheath as characteristic of animal chromosomes and possibly also of plant chromosomes, seems to us to be open to serious question, at least in the form stated. It has been considered at length in a recent paper by de Litardière ('21), who interprets the "sheath" as an artifact—probably a fixation product. The latter author maintains that the same sort of zone is to be seen around the mitochondria, cytoplasmic inclusions, etc., and hence has no special connection with chromosomes. We may omit further consideration of these structures, therefore, until the evidence becomes clearer.

Possibly de Litardière's criticism may apply to the cases we have cited here of "indications" of a hyaline zone around chromosomes in the Diptera, including *spermatogonial* chromosomes of *Lasiopogon;* and it may apply to the zone seen in McClung's figures referred to above. Further evidence is required to settle this point. But we may note that in these cases the zone seems to be found only around the chromosomes, not around other structures in the cell.

It may be added that in the case of the *spermatocytes* of *Lasiopogon* described here the structures are clearly not artifacts, and are confined to the chromosomes (including the nucleolus which, from analogy with other Diptera, is assumed to be chromosomal in nature). Numerous large mitochondrial rods are present, but are not surrounded by sheaths comparable to those enveloping the chromosomes.

If we omit the questionable cases, such as those just mentioned as possibly due to artifacts, it is clear that few forms exhibit structures comparable to those in *Lasiopogon*, although the possibility still remains that the structures may be *present* in others even when not visible.

In the foregoing discussion little emphasis has been laid on the fact that some of the cases considered involve bivalent chromosomes (maturation stages) and others univalent chromosomes. It is not yet clear that any distinction should be made, so far as the present subject is concerned. But it may be noted in closing that any view which considers the chromosome as enclosed in a sheath or envelope must take account of the approximation of

homologous chromosomes during synapsis, and, in the case of the Diptera, must allow for such an approximation in every cell generation (cf. Metz, '16) This would seem to necessitate the assumption that the envelopes of homologous chromosomes could unite.

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## EXPLANATION OF FIGURES.

The figures were drawn with the aid of a camera lucida, using a Zeiss 2 mm. oil immersion objective and number 12 ocular. Magnification about 1000 diameters.

All figures except number 4 are from specimen number 2056.

It should be emphasized that the figures represent cross sections at *one level* in most cases, and do not represent entire nuclei.

FIG. 1. Spermatogonial metaphase, probably not entire.

FIGS. 2–10. First spermatocyte growth period.

FIG. 2. Very early growth stage.

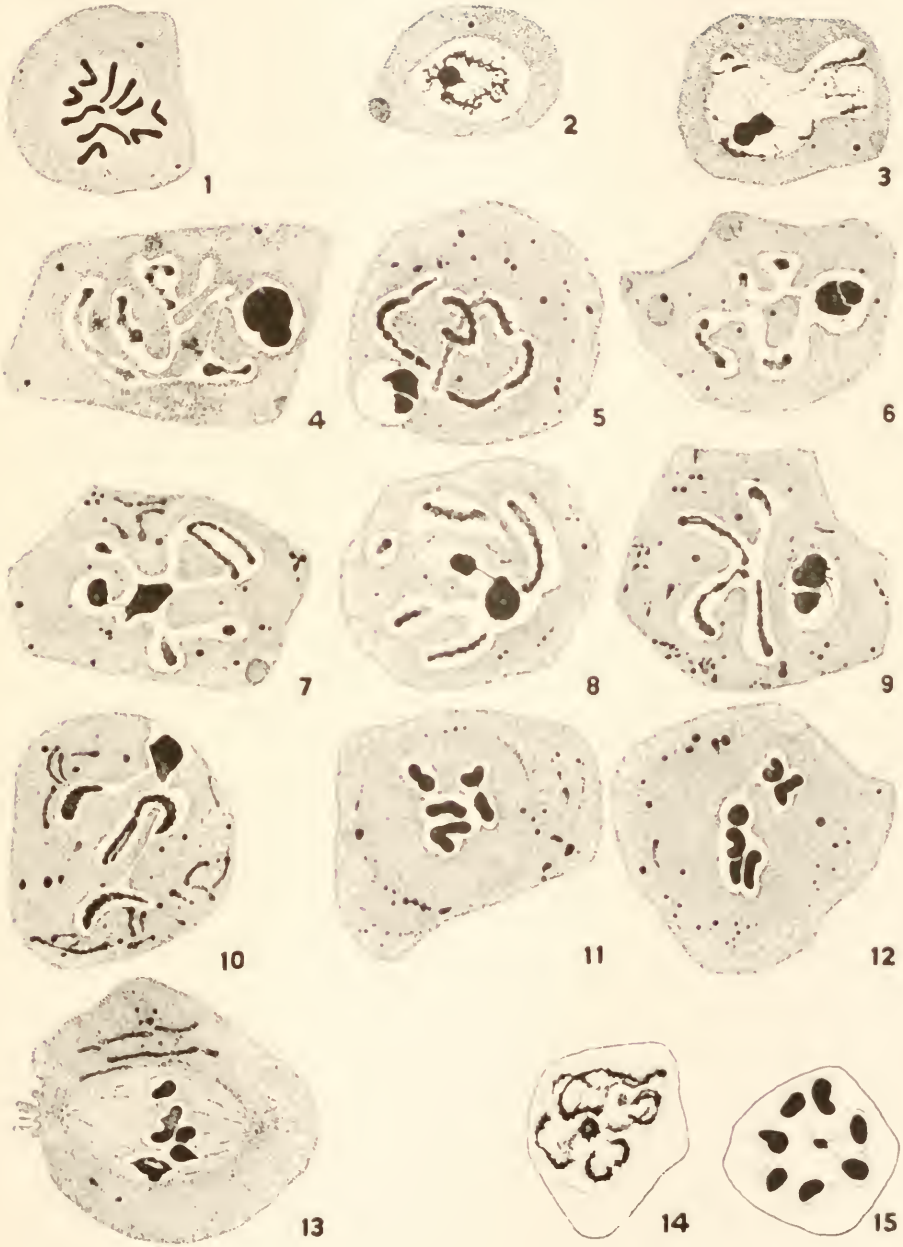
FIG. 3. Slightly later, nucleus becoming lobulated.

FIG. 4. Later, but still in the early part of the growth period; nucleus completely transformed into narrow ramifying chambers.

FIGS. 5–10. Various aspects of the nuclei during the successively later growth stages, showing the lobes and chromosomes cut longitudinally, transversely and at intermediate angles. The isolated portions in figures 8 and 9 are sections of lobes, not separate vesicles.

FIGS. 11–13. First spermatocyte metaphase in polar and lateral views, showing the persistence of the hyaline cortical zone.

FIGS. 14–15. Ptecticus (see text, p. 159).




C. W. METZ AND J. F. NONIDEZ

# THE SUSCEPTIBILITY OF CELLS TO RADIUM RADIATIONS.

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It has frequently been observed that cells may show at different periods of their existence marked variations in susceptibility to radium radiations. An embryonic cell is more quickly injured than the same cell in the adult condition: at the metaphase, cells are more sensitive than they are immediately before or after that brief period (1). Bohn (2) first suggested that the underlying cause of such differences in response must be sought in the physiological condition of the cells at the time of radiation. That they cannot be due to changes in the absorptive power of protoplasm is obvious: whether a cell is sensitive or not, the rays are absorbed to the same extent. The actual changes produced by them in protoplasm must therefore be the same. But the reaction of the cell to such changes differs immensely. We may therefore say that a cell is susceptible when it is in such a physiological condition that a modification produced by the rays results in a greater or less injury, and that it is resistant when the same modification is not followed by injurious effects.

The object of the present paper is to show that among the conditions which affect the susceptibility of cells to radium radiations are (1) the temperature of the cells at the time of exposure, and (2) the relative permeability of the surface layer of the cell.

The experiments to be described were carried out on certain Protozoa, for these cells are better adapted to this kind of experimentation than any others. They can live in both high and low temperatures without injury: and different genera show marked differences in permeability. Cells of the same species, even descendants of the same individual, vary widely in their reaction to radium radiations at different periods of their life cycle. *Paramœcium* is perhaps the best cell for experimental purposes since it is more susceptible than any other common type.

I will not here review the results obtained by other investigators who have tested the effects of radium radiations and X-rays on Protozoa. Different methods of exposing the cells lead to such wide variations in results that comparisons cannot safely be made. In general, however, cells which are sensitive to the one are also sensitive to the other type of radiation. Great differences in the susceptibility of cells of the same species have been reported. Thus Zuelzer (3) states that *Pelomyxa palustris* when exposed to 6 mg. of radium element dies sometimes within one hour and sometimes only after four hours of continuous exposure. Such differences make any conclusion as to the length of the lethal dose out of the question. But with appropriate methods the lethal dose is found to be constant.

## Methods.

The method used in the following experiments is this. The radium was enclosed in a glass capsule which prevented the alpha rays from escaping. In the experiments on the relation of temperature and permeability to susceptibility, the strength equalled 13.4 mg. of element. In the third series, on the change in permeability induced by the radiations, the strength was 25 mg. of element. The radium tube was used unscreened and was supported above the drop of culture medium at a distance of 2 mm. Thus all of the rays which emerged from the lower side of the tube could reach the cells. The whole preparation was kept in a moist chamber at the desired temperature.

In order to determine what type of rays produced the effects which are to be described, I interposed between the radium tube and the *Paramœcia* lead sheets of various thicknesses, thus filtering out the more penetrating rays. All of the beta rays are stopped by 2 mm. of lead: the gamma rays are not affected. It became apparent at once that the changes produced in the *Paramœcia* were due to the action of the slowest beta rays, for when a lead screen of 0.12 mm. was interposed the *Paramœcia* were affected hardly at all. This is to be expected, for the surface layer of the cells is very thin and can absorb only those rays which have a low velocity: it offers almost no resistance to rays having considerable powers of penetration.

In conducting these experiments it was found necessary to use

only *Paramœcia* from a pure culture, for unrelated wild cells show great variations in their susceptibility to the rays. Another necessary condition has been mentioned by Jacobs (4), namely, that in each test the same amount of liquid must be used.

### The Reaction of Paramœcium to Radium Radiations.

When a *Paramœcium* is exposed to radium radiations under the conditions described, it quickens its movements at first and then gradually slows down and ceases to swim unless the dish is shaken. Later the contractile vacuoles pulsate more and more slowly and finally stop, usually in the expanded condition. If radiation is longer continued, a typical cytolysis ensues. The cells imbibe water, swelling considerably in consequence, and the ectoplasm bulges out in the form of clear vesicles which later run together. Then the pellicle separates from the rest of the cell carrying with it the cilia. The protoplasm is now highly fluid. At this time the macronucleus, in stained preparations, is seen to be divided into several parts. Not infrequently the cells burst violently. These phenomena are in every point similar to those which are observed when *Paramœcium* is treated with a variety of cytolytic agents, as described by Budgett (5), Harvey (6), and Jacobs (4).

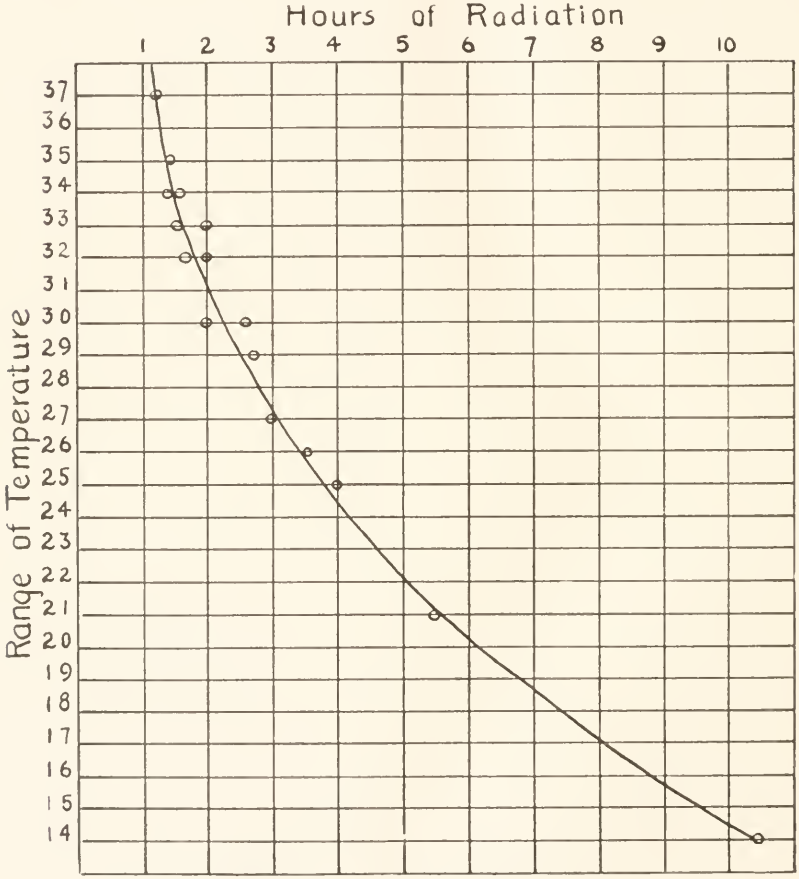
### The Relation of Temperature to Susceptibility.

Rhodenburg and Prime (7) first pointed out that there is a definite correlation between temperature and the susceptibility of cells when treated with X-rays. In their experiments they exposed mouse sarcoma in vitro at a temperature of 42° C. to a definite dose of X-rays, and then inoculated healthy mice with the radiated cells. At this temperature 10 per cent. of the inoculations failed to take. When the cells were radiated at 43° C., 76 per cent. of the inoculations failed. Control experiments proved that these temperatures alone are not sufficient to produce this effect. The combination of high but sublethal temperatures with radiation was five times as effective as radiation alone.

Mammalian tissue cannot be subjected to wide variations in temperature, but the Protozoa can live normally at temperatures as low as 15° C. and as high as 37° C. In the following experiments these were the limits employed.

When *Paramœcia* are radiated at high temperatures, they suc-

cumb much more quickly than at low temperatures. The results are shown in the accompanying figure. An analysis of the curve shows that for each increase of about 8° C. the length of the lethal dose is halved. The curve is thus similar to that which expresses the relation between temperature and the velocity of a great number of reactions both inorganic and physiological.




TEXT-FIGURE 1. The effect of temperature on susceptibility.

Snyder (8) cites more than fifty physiological reactions which conform to this type of curve: Woodruff and Baitsell (9) show also that the division rate of *Paramœcium aurelia* varies similarly with changing temperature.

The cells were not injured by these temperatures alone. While

the experiments were in progress the room temperature varied from 30° to 36° C. and the cultures flourished. Single lines of cells showed a steady division rate of about 2½ divisions per day. In the coolest temperatures the cells remained normal, and regained the usual division rate on being brought back into a warmer place.

This increase in susceptibility at high temperatures is not due to any increased activity of the radiations, nor to any change in the power of protoplasm to absorb the rays. The amount of radiation absorbed is determined by the atomic constitution of protoplasm and this does not vary materially during changes in temperature. One of the conditions which varies with the temperature is the permeability of the cell membrane. Hober (10) states that the permeability of plant cells is doubled with each increase of 10° C., the cells being eight times as permeable at 30° as they are at 0° C. That *Paramœcia* are more permeable in warm than in cold solutions can be demonstrated by staining them at 30° and 14° C. in neutral red. In the higher temperature they stain deeply: at the lower, they do not stain at all. Whether a change in permeability is the only cause of increased sensitiveness to radiations remains to be demonstrated. That it is an important factor is shown in the next section.

### The Relation of Permeability to Susceptibility.

The fact that cells are most sensitive to radiations when their permeability is increased by heat suggests that the two phenomena are related. Here again the Protozoa are admirably adapted for testing this point, for the permeability of the cell membrane can be measured in the living condition. The method employed in these experiments is that followed by Harvey (6). A number of *Paramœcia* from a pure culture are drawn up into a capillary pipette which is calibrated so that exactly the same amount of liquid is taken in each experiment. The cells are stained for ten minutes in a solution of 0.02 per cent. neutral red mixed with 10 cc. of tap water. At the end of this time the vacuoles at the posterior end of the cells are a bright pink. The surrounding protoplasm is also colored. The neutral red in this dilution is not toxic, although in more concentrated solutions it produces cytolysis.

The cells are now drawn up in a calibrated pipette and added

to exactly 2 cc. of $n/1280$ $NH_4OH$ solution. The amount of liquid thus added reduces the strength of the ammonia to $n/1300$. The stained *Paramœcia* when put into this solution give first the avoiding reaction and quickly begin to lose color. The protoplasm, and later the gastric vacuoles, turn yellow, and finally become colorless. There is a wide variation in the rate at which the color fades in individual cells, some destaining in three or four minutes while others retain some pink color as long as ten minutes. In order to determine the average time for destaining the usual number of cells (about 40), I used the following method. The cells were observed, during their destaining, under a binocular microscope and each one, as soon as it lost color, was removed and the time which had elapsed since its first entrance into the ammonia solution recorded. The following measurements, typical of many, indicate the rate of destaining.

PARAMŒCIUM DESTAINED IN $n/1280$ $NH_4OH$.

| Minutes Elapsed. | No. of Cells Destained. |
|---|---|
| 1 | 0 |
| 2 | 0 |
| 3 | 0 |
| 4 | 3 |
| 5 | 2 |
| 6 | 4 |
| 7 | 5 |
| 8 | 8 |
| 9 | 6 |
| 10 | 6 |
| 11 | 6 |
| 12 | 5 |
| | Total 45 Ave. 8.5 min. |

By this method the personal equation is greatly reduced since the observer cannot form any idea of what the average will be until the entire number of cells has been destained. Many tests on the same pure culture of *Paramœcium* gave very constant results, the average time of destaining in tests carried out on the same day varying less than one half minute. Other lines of *Paramœcia* showed somewhat different averages but even here they differed from each other by not more than one and one half minutes.

A study of *Paramœcium* cells at different phases of their life cycle shows that the permeability varies, being much greater at the time of conjugation than at any other period. Thus among four lines of cells in which conjugation did not occur, the de-

staining time varied from 7.8 minutes to 9 minutes. In a culture which was undergoing an epidemic of conjugation the pairs showed an average destaining time of 4.5 minutes. This culture was presumably not pure, and the pairs in their reaction to the ammonia showed a wider variation than was found in any homogeneous group. But the difference in permeability between conjugants and non-conjugants was in every case large enough to be significant.

Other Protozoa differ from *Paramœcium* in permeability. As Harvey has pointed out, *Stylonichia* and *Oxytricha* are comparatively impermeable. Indeed, after ten minutes in neutral red of the usual concentration they have taken up almost no color at all. The stain must act for twenty-five to thirty-five minutes before these cells are stained sufficiently for experimental purposes, and even after this time they are not as highly colored as *Paramœcium* is after ten minutes. The time required for destaining *Stylonichia* varies somewhat in different cultures, but the average is approximately forty minutes.

When the relative permeability of these cells is compared with the length of their lethal dose of radium radiations, we find a close correlation between the two measurements: that is, cells which are relatively permeable are quickly killed by the rays, while those which are less so are more resistant. The following table indicates these relations.

COMPARISON OF THE DESTAINING TIME AND LETHAL DOSE OF RADIUM RADIATIONS AT 27° C.

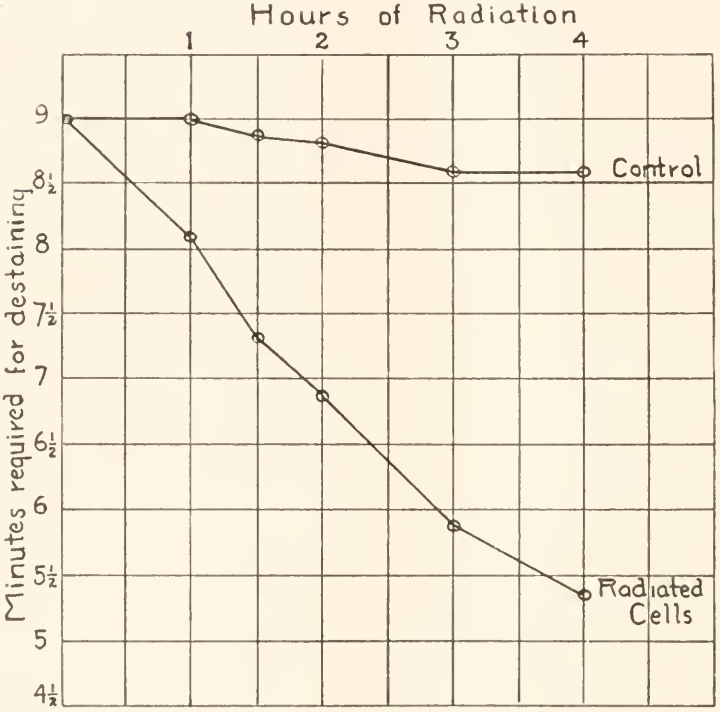
| | Destaining Time. | Lethal Dose. |
|---|---|---|
| *Paramœcium* single cells | 8.6 min. | 3 hours |
| *Paramœcium* conjugating | 4.5 min. | 1½ hours |
| *Stylonichia* | 40. min. | 15 hours |

It is evident therefore that the susceptibility of these Protozoa to radium radiations varies directly with the permeability of the surface layer of the cell.

## THE EFFECT OF RADIUM RADIATIONS ON THE CELL MEMBRANE.

The question naturally arises, what is the reason for this correlation? The answer is to be found in the fact that the rays which are absorbed produce in the cell membrane changes which lead to increased permeability. The experiments described below indicate the rate at which these changes take place.

In the following experiments the radium amounted to 25 mg. of element. The cells were exposed in the same manner as before, then stained in neutral red and destained in $n/1280$ $NH_4OH$. The accompanying figure shows the results of experiments per-



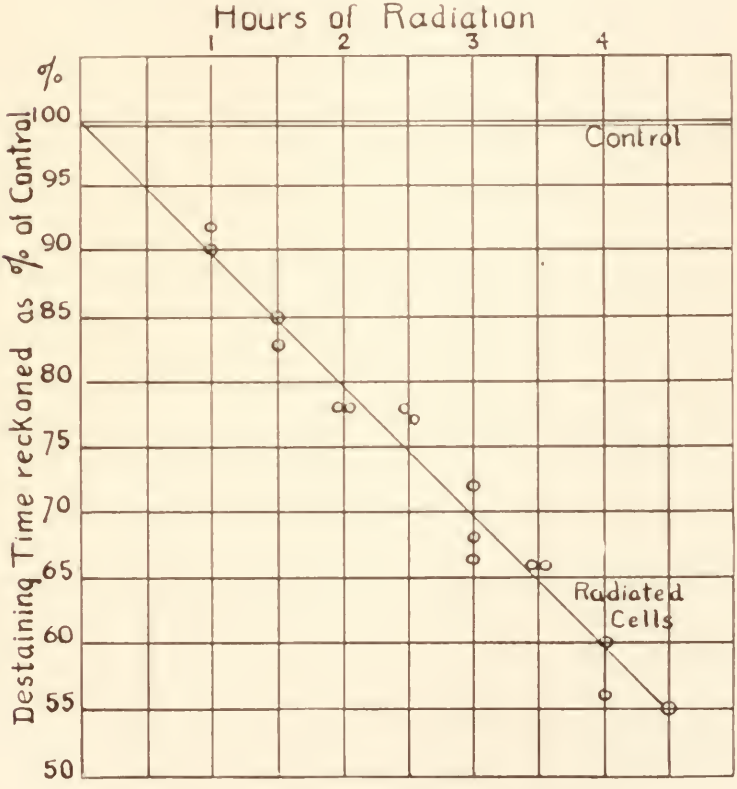

TEXT-FIGURE 2. The effect of radiations on permeability.

formed in one day. It is necessary to make many tests within the limits of a few hours for the cells vary somewhat in their reactions to these manipulations with changing conditions of food, etc. By using two tubes, each amounting to 25 mg. of element it was possible to perform two experiments simultaneously and thus make seven or eight determinations in a single day. The temperature in these tests remained uniformly at 22° C.

The control cells showed a slight increase in permeability after remaining in a small drop of culture medium for some hours. This however is not significant, for cells left as long as eight hours show practically the same reaction to ammonia as those

left for only two hours in a small drop. But the radiated cells display a marked shortening in the time required for destaining, or, in other words, they show a considerable increase in permeability, the change beginning very soon after the exposure to radium commences. At the end of four hours the cells are still alive but are for the most part motionless. If the exposure is longer continued the cells die, showing the characteristic signs of cytolysis.

The second figure shows the result of a larger number of tests.




TEXT-FIGURE 3. The effect of radiation on permeability.

Since they were made during the course of many weeks the controls varied somewhat, although on any one day they were very uniform. The time required for destaining is here given in percentages of the control time which is reckoned as 100 per cent. The curve clearly indicates that the normal semi-permeability of

*Paramœcium* increases progressively as the exposure is more and more prolonged.

Some of the radiated cells were removed to separate drops of culture medium before treatment with the stain, and their division rate observed. Those which were exposed for one hour or a little less frequently showed a higher rate than the controls. This result has been described by Markowits (11) who studied the effect of mesothorium rays on *Paramœcium*. The acceleration can be observed for five or six generations, after which the cells return to normal and show no evidences of injury. The same phenomenon I have observed in sea urchin eggs when lightly radiated (12). Indeed, a stimulation in the rate of growth is of general occurrence, having been noticed in the case of growing plants, embryos, tissue cultures, and abnormal tissue growths.

From these facts we may conclude that the slowest beta rays increase the permeability of the surface layer of *Paramœcium* cells. If the exposure is brief, this change in permeability is accompanied by an acceleration in the rate of cell division. If it is more prolonged, a destructive cytolysis ensues, and the cells die.

## Discussion.

Radiations which are absorbed at the surface of the cell produce definite changes which lead to an increase in permeability, and, if the exposure is sufficiently prolonged, to complete cytolysis. For a definite dose, such changes must be the same in extent, regardless of the physiological condition of the cell, for the absorptive power of protoplasm remains constant. Yet it is quite apparent that a given dose of radiation may result in no appreciable injury in certain instances, while in others it is followed by the death of the cells. This is clearly shown in the text-figures. For example, at 22° C. radiated *Paramœcia* undergo cytolysis, under the conditions described, in five hours. From the beginning of the exposure to the death of the cell there is a steady increase in permeability. An exposure of half this duration is followed by no permanent injury. But when cells are radiated at 30° C. they are cytolyzed after only two and one half hours. Cytolysis occurs when the permeability of the cell has been raised above a definite limit. If it is already high, due to high temperature or to other conditions, the cytolytic action of

the rays quickly raises the permeability above the limit and the cell dies. But if it is low, the lethal point is reached only after a prolonged exposure.

A similar phenomenon is observed in eggs exposed during different phases of mitosis. At the metaphase their permeability, as shown by Lyon (13) and others, is notably greater than at any other period: so also is their susceptibility. I have shown (12) that an amount of radiation which will induce a quickened cell division in sea urchin eggs, when applied just before or after the metaphase, has a retarding effect when applied at that period. That is, the cells are more sensitive then than they are during the prophase or telophase of mitosis.

The same results are obtained when other cytolytic agents are used in place of radium radiations. Lillie (14) finds that if freshly fertilized sea urchin eggs, which are highly impermeable, are treated with hypotonic sea water they resist its cytolytic action for thirty minutes. But if they are placed in this solution when the cleavage furrow appears, they rapidly undergo cytolysis.

It appears possible therefore that the susceptibility of cells may be raised by the simple expedient of increasing their permeability by heat or by some other means. This has been done by Rhodenburg and Prime (7) in the experiments already cited. How far this method can be used in the treatment of abnormal tissue growths remains to be demonstrated.

The fact that agents which differ so widely as do radium radiations and hypotonic sea water produce the same effects under similar conditions suggests that the action of these rays, and also other types of radiant energy, such as ultra-violet light and alpha rays, is not peculiar to themselves. Indeed it may be said that any of the rays which are absorbed at the surface of the cell will cause changes differing in no way from those produced by a great variety of chemical cytolytic agents.

The more penetrating beta rays, the gamma, and X-rays appear to be so slightly absorbed at the surface of cells which are freely exposed to them that they can produce little or no effect. Richards (15) tested the permeability of various eggs and of *Arenicola* larvæ after an exposure to X-rays and found no evidence of any increase. I have shown that the rapid beta and the gamma rays of radium do not act on the surface layer of *Paramœcium*.

But it has been demonstrated that very definite proportions of these rays are actually absorbed by cells of deep-lying tissues. And histological study shows that such cells after radiation imbibe water, swell considerably, and undergo degenerative changes which have all the appearance of a typical cytolysis. It is apparent, then, that any kind of rays which are absorbed produce the same effects. But these more penetrating rays differ from the less penetrating types in this respect, that they are also able to bring about degenerative changes in the interior of the cell, particularly in the nucleus, which the latter, because of their slight penetrating power, are unable to produce.

I have mentioned the fact that a brief radiation with the slow beta rays produces an acceleration in the division rate of *Paramœcium*. This effect is not restricted to the action of these particular rays, for all the radiations of radium, as well as X-rays, can produce this result if the proper exposure is made. This has been demonstrated in a great variety of cases, some of which have already been cited. According to Lillie (16) such acceleration is the direct outcome of increased permeability, for this condition allows a freer interchange of $CO_2$ and Ó through the surface layer and a consequent hastening of all metabolic activities. It may be possible, therefore, that all cases of stimulation following exposure to radium radiations and to X-rays can be explained on this basis.

## Summary.

1. The susceptibility of *Paramœcium* to radium radiations (chiefly the slowest beta rays) varies with the temperature at the same rate as do physiological reactions of various kinds.

2. The susceptibility also varies directly with the degree of permeability of the surface layer of the cell.

3. The slow beta rays act on the surface layer of the cell, increasing its permeability, and if allowed to act long enough, causing a typical cytolysis. In this respect they resemble other types of radiant energy, and diverse chemical cytolytic agents.

4. Cells which have a relatively high permeability are more susceptible than those having low permeability, for the cytolytic action of the rays is quickly followed in the former by a cytolysis which is irreversible, while in the latter it is reversible.

5. It is suggested that this increase in permeability, following brief exposures, is the cause of the acceleration in division rate seen in *Paramœcium* and in other cells and tissues.

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# THE PHYSIOLOGICAL AND SYMBIOTIC RELATIONSHIPS BETWEEN THE INTESTINAL PROTOZOA OF TERMITES AND THEIR HOST, WITH SPECIAL REFERENCE TO *RETICULITERMES FLAVIPES* KOLLAR.*

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This is the second of a series of papers on this subject. The first paper of the series, "Correlation between the Food and Morphology of Termites and the Presence of Intestinal Protozoa," deals with the more general aspects of the subject, and will appear in the July (1923) number of the *American Journal of Hygiene*.

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## INTRODUCTION.

The most unusual and peculiarly interesting faunal association known to parasitology is that represented by the teeming menagerie of intestinal protozoa harbored by termites. These protozoa are favorite objects for study and have attracted the attention of many investigators. The initial microscopic view of thousands of these organisms, but recently removed from the termite's intestine, wriggling, undulating, and twisting in a drop of saline solution, never fails to fill the observer with enthusiasm, and bring forth volleys of questions. Perhaps the first thing to attract attention is the abundance, both in form and number, of protozoa present in a single host. The large abdomen is almost completely filled by the greatly distended intestine, the lumen of which is gorged by a vast horde of protozoa.

The protozoa of the termites, for the most part, differ considerably from those found in other animals, and this difference cannot fail to attract the attention of a protozoölogist. Their most distinctive features are: the ingestion of solid particles of wood for food; the diversity of organization among the various forms; the degree of structural specialization [1] attained by many of the species, resulting in the formation of the most complex flagellate organelles yet known.

No observer has ever failed to raise the question of the relation of the protozoa to their host, but most investigators in the past have regarded the protozoa either as true parasites or commensals, though, in almost every instance, no proof whatever is offered in support of either contention.

At present no termite of the most highly specialized family, the Termitidæ (Metatermitidæ Holmgren), is known to harbor intestinal protozoa; but all the genera and species of the other families, Kalotermitidæ (Protermitidæ Holmgren), Rhinotermitidæ (Mesotermitidæ Holmgren) and Mastotermitidæ, that have been examined [2] have been found to harbor enormous

[1] See especially the papers of Kofoid and Swezy (1919) describing the neuromotor system of *Trichonympha campanula* and *Trichomitus termitidis*.

[2] See the first paper of this series, "Correlation between the Food and Mor-

numbers of protozoa. It is not definitely known why protozoa are never found in the intestine of any of the Termitidæ; but a difference in the feeding habit has been suggested as a very probable explanation, since few, if any, of the Termitidæ feed solely on wood.

## THE PROBLEM.

To determine the relation of the intestinal protozoa of termites to their host.

Experimental methods were the paramount mode of attack; and it was necessary, in order to solve the large problem stated above, to solve several problems, which may be briefly stated as follows:

1. What is the principal compound in wood, the sole article of diet of most termites, which is used for food.

2. How do termites utilize cellulose, the principal compound in wood, as their chief article of food?

3. How may all of the intestinal protozoa be killed without at the same time injuring their host? To develop a rapid and successful method for removing the protozoa from the termites was a very difficult proposition.

4. Why do termites, when subjected to a temperature of 36° C. for 24 hours, lose their ability to maintain themselves on a wood diet?

5. Is defaunation (the removal of the protozoan fauna), which occurs when termites are incubated at 36° C. for 24 hours, in any way responsible for the inability of the termites to maintain themselves on a wood diet?

6. Why do the incubated and defaunated termites live indefinitely when fed the decomposition products of wood, or the products of fungus-digested cellulose; but die within 10–20 days when fed wood, their normal diet, or when fed cellulose?

7. Why do the incubated and defaunated termites regain their ability to make use of wood or pure cellulose as food when reinfected (refaunated) with protozoa?

8. Do the unincubated and faunated termites harbor any in-

phology of Termites and the Presence of Intestinal Protozoa," for a list of the genera and species that have been examined. Since writing this more than 75 species of the Termitidæ have been examined and three of this number have been found to harbor protozoa and feed on wood.

testinal bacteria or fungi, capable of digesting cellulose, which are killed off during incubation?

9. Do the protozoa aid their host mechanically to digest wood, or do the protozoa digest the wood particles which they take into their bodies and convert them into substances which their host uses for food?

10. Since the termites lose their ability to digest wood when the protozoa are removed from their intestine, that is when they are defaunated, but regain this ability when reinfected with protozoa, and since the protozoa themselves can be shown to digest the wood particles which they take into their bodies, does a true symbiotic relationship exist between termites and their intestinal protozoa?

## MATERIAL.

*Reticulitermes flavipes* Kollar, collected in Maryland, near Baltimore, was used for all the experimental work on this genus, though many colonies from other states have been used for studying the protozoa of this species. *Termopsis* sp. (the species could not be determined because there were no winged adults in any of the colonies collected, but it is either *angusticollis* Hagen or *nevadensis* Hagen) from Ashland, Oregon, owing to its large size, was used in many of the feeding experiments, though *Reticulitermes flavipes* was also used in all of the feeding experiments. *Kalotermes schwarzi* Banks, *K. jouteli* Banks and *Prorhinotermes simplex* Hagen, from southern Florida, were used for incubation experiments, but the material of these genera was not sufficient to make possible as extensive a study as was carried out with the material from Maryland. *Amitermes* sp., from Uvalde, Texas, and *Nasutitermes morio* Latreille, from Porto Rico, both of the family Termitidæ, were studied.

## ACKNOWLEDGMENTS.

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## GENERAL CONSIDERATIONS.

### 1. The Termites.

#### *a. Castes.*

In order that the reader, unfamiliar with the nature of a termite colony, may know something of the castes present, the following condensed statement (taken from the papers of Snyder and Thompson) is made. In *Reticulitermes flavipes* there are five castes, three of which are fertile or reproductive, and two that are sterile or non-reproductive. Males and females occur in each of the five castes. The reproductive castes are: (1) the *first form*, which has three well-defined phases of development: (*a*) the nymphs, with long wing pads, creamy white body 1.3–1.4 mm. long, and light brown eyes; (*b*) the winged adults, with long wings, dark brown body 6 mm. long, and black eyes; (*c*) the older males and females, with enlarged abdomens and the scales of the shed wings, body 7–14 mm. long: (2) the *second form*, which, like the first form, has three well defined phases of development: (*a*) the nymphs, with short wing pads and colorless body and eyes; (*b*) the young adults, with short scaly wing vestiges, straw-colored or grayish body 6–7 mm. long; (*c*) the older adults, with wing vestiges, enlarged abdomen, body length 7–12 mm: (3) the *third form*, which also has three developmental phases: (*a*) the nymphs, wingless, with white head and body, and eyes that are invisible in the living or unstained specimen; (*b*) the young adults, wingless, head and body pure white, opaque and not transparent, about 6 mm. long; (*c*) the older adults, with enlarged abdomen, wingless, head and body white, 7–9 mm. long. The other castes, the sterile castes, are: (4) the *worker*, wingless with grayish abdomen, only two developmental phases, *i.e.*, nymphs and adults, always feeds on wood and there is no degeneration of the jaw muscles, always harbors protozoa once an infestation has been acquired, salivary glands small and very little fatty tissue is present in the body; (5) *soldier*, wingless with elongated head covered with thick yellowish chitin, mandibles dark brown long slender and curved, abdomen shorter than in other castes and more flattened, nymphs and adults only, no post adult growth.

Thompson (1917) showed that the newly hatched nymphs of

*Reticulitermes flavipes*, 1.1 mm. long, although externally all alike, could be differentiated by their internal structures into two distinct types, namely, (*a*) reproductive nymphs, from which develop the three fertile adult castes, and (*b*) the worker-soldier nymphs, from which develop the two sterile adult castes. By the time the reproductive nymphs had attained a body length of 1.3–1.4 mm. they could be differentiated by their internal characters into nymphs of the first form and nymphs of the second form, which developed, finally, into the two respective reproductive adult castes. Soldier and worker nymphs could be differentiated, internally, when they had attained a body length of 3.75 mm. Nymphs of the third form could not be differentiated until a body length of 4 mm. was attained.

Several intermediate forms have been discovered by various investigators, intermediates between the first and second forms, three fertile soldiers (Heath, 1902)[1], and others; but these need not be considered here.

The five castes occur in most termites. Known exceptions are as follows: the third form reproductive caste occurs in few, if any, of the Termitidæ; in fact none have been reported from the Nearctic genera of this family, and it is doubtful if this caste occurs in any genus of the Termitidæ. Apparently this caste has been lost in this, the most highly specialized, family of termites. The third form is less common than the first and second in *Reticulitermes flavipes*, but in *Termopsis* it is more common than the first and second form. *Reticulitermes* is about midway between the most primitive and the most highly specialized termites. *Termopsis* is a primitive genus. A true worker caste is not present in *Termopsis* and *Neotermes*, but a large-headed worker-like reproductive form is present. Two genera, *Kalotermes* and *Cryptotermes*, have no worker caste. The genus *Anoplotermes* has no soldier caste.

Grassi calls the first forms "true" or "perfect insects," or

[1] Wheeler (Social Life among the Insects, Scien. Monthly, 16, 1923, p. 172) says: "The soldiers and workers are normally sterile, but sometimes they become fertile and probably on such occasions reproduce their own castes." Snyder (personal communication) says Heath now admits he was wrong. Also experiments which I have conducted recently reveal the fact that the soldiers of at least several, and probably all, species of xylophagus termites cannot live to themselves when given their normal diet of wood.

"the royal forms." The second and third forms he calls "substitute" and "complemental" forms, which forms he thinks are always ready to take the place of the royal forms in case of need. He recognizes nymphs of the first and second forms, but these, he thinks, if taken young enough and given the proper food, may be changed into anything else, *i.e.*, any of the castes. But, Thompson (1919 and 1922) and Snyder (1920) have presented valuable experimental evidence in support of the view that each of the three reproductive castes breeds true, that is, produces reproductive adults only like itself.[1]

*b. Food—Direct.*

Wood is a direct food of most termites. Some of them feed on dry heartwood, while others feed on moist and more decayed wood, usually sapwood. *Kalotermes schwarzi*, for instance, is often found in furniture stored in an attic, since it does not require access to the soil in nature as does *Reticulitermes flavipes*. However, it is quite probable that *R. flavipes* requires access to the soil only in order to obtain moisture, since it may be kept in captivity indefinitely without access to soil provided sufficient moisture is given it. Water, of course, is a direct food, though some termites require very little of it. *Amitermes tubiformans* Buckley, for example, feeds principally on dry cow chips and soil, but occasionally eats the roots of grass and other vegetation. The manure is often carried down into chambers below the ground. Many of the Termitidæ feed on plants, sometimes attacking flowers, shrubs and field crops. Fungus gardens are cultivated by many speices of this family. These termites carry large quantities of vegetation into their huge nests and take advantage of the digestive action of the fungus on the hemicellulose, the fungus and hemicellulose being eaten by them as food. It is interesting to note that leaf-cutting ants (*Œcodoma*) do the same thing. They carry thousands of leaves to their nest, where they use them to make a compost heap, on which fungi, to which the ants are very partial, grow. Termites in captivity may be induced to eat almost anything, but our knowledge regarding

[1] Each of the adult sexual forms can reproduce itself and the forms below it in rank. Thus, the first form adults reproduce all castes: the second form individuals like themselves, third form, soldiers and workers; the third form individuals like themselves, soldiers and workers.

their habits in nature, especially the Termitidæ, is so limited that it is impossible to adequately discuss their food.

*c. Food—Indirect.*

The liquid diet is either a salivary secretion or regurgitated material from the mid-gut. It is very probably the former. It is a colorless and distinctly alkaline liquid which collects on the labrum as a small drop, which may be used either as food for others or for the termite secreting it. Many termites also employ this liquid in building. In *Reticulitermes flavipes* the young larvæ of all the castes and the second and third forms during the third developmental stage feed solely on this liquid diet which they receive from other members of the colony. Later, the larvæ of all the castes eat the excreta as it is passed from the anus of the older members of the colony, and it is at this time and in this way that they first become infected with protozoa. The larvæ also begin to eat wood at or about the same time that they begin to eat excrement—which in this termite is composed of a few wood particles, but primarily of protozoa and liquid. The soldiers, workers and first form reproductive males and females always feed on wood, though they—especially the first forms during the third developmental stage—partake of a liquid diet as well. The exuviæ are usually eaten and, sometimes, the dead bodies of other members of the colony are eaten.

2. The Protozoa.

Lespes (1856) made the first recorded observation of termite protozoa, though he did not describe them. Leidy (1877 and 1881) was the first in America and the second in the world to observe these organisms. In 1881 he gave descriptions of the protozoa which he had observed in *Reticulitermes flavipes*, creating three genera for them, *Trichonympha*, *Pyrsonympha* and *Dinenympha*. This termite really harbors several other genera, most of which were included in Leidy's drawings as stages in the life cycle of the three genera mentioned above. More than a score of other investigators have studied these protozoa, and to date 41 genera have been described. For a complete account of the work done by all the students of termite protozoa and for a classification of all the protozoa which have been described from

termites the reader is referred to the first article of this series, "Correlation between the Food and Morphology of Termites and the Presence of Intestinal Protozoa."

All of the known termite protozoa, except *Gregarina termitis* and *Nyctotherus termitis*, have been placed in two orders of flagellates, the *Polymastigina* and the *Hypermastigina*, the latter being the most highly specialized order of flagellate protozoa.

### 3. Biochemical Considerations.

Dore (1920) gives the following analysis of yellow pine (*Pinus ponderosa*) oven-dry (100° C.) basis. Results in percentages.

| | | | |
|---|---|---|---|
| Benzene extract | 2.22 | Lignin | 29.47 |
| Alcohol extract | 1.49 | Xylan | 3.49 |
| Cellulose | 57.72 | Mannan | 6.37 |
| | | Galactan | 0.78 |

The two following examples are taken from Ritter and Fleck (1922), who have analyzed a large number of American woods:

Analysis of a mean of four samples. Results expressed in percentages of oven-dry (105° C.) material.

| | Western Yellow Pine (*Pinus ponderosa*). | Tanbark Oak (*Quercus densiflora*). |
|---|---|---|
| Moisture | 6.42 | 3.66 |
| Ash | 0.46 | 0.83 |
| Solubility in cold water | 4.09 | 4.10 |
| Solubility in hot water | 5.05 | 5.60 |
| Solubility in ether | 8.52 | 0.80 |
| Solubility in 1% NaOH | 20.30 | 23.96 |
| Acetic acid | 1.09 | 5.23 |
| Methoxy | 4.49 | 5.74 |
| Pentosan | 7.35 | 19.59 |
| Methyl pentosan | 1.62 | none |
| Lignin | 26.65 | 24.85 |
| Cellulose | 57.41 | 58.03 |
| In the cellulose: | | |
| Pentosan | 6.82 | 22.82 |
| Methyl pentosan | 1.98 | none |
| *Alpha*-cellulose | 62.10 | 56.77 |
| *Beta*-cellulose | 10.56 | 19.92 |
| *Gamma*-cellulose | 30.13 | 23.93 |

That the principal food of most termites is cellulose is a fact which appears to have escaped the attention of physiologists and biochemists. Because of this, and also owing to the fact that our

text-books of chemistry and physiology give little, if any, of the information which has been gained in recent researches on the chemistry and digestion of cellulose, it seems expedient to briefly mention—with no pretention at a discussion—some of the salient features brought out by a few of the more important of these researches.

According to Hibbert (1921) "cellulose is nothing more than a polmerized dextrose glucoside of dextrose," and Irvin and Hirst (1922) in their most recent paper on the constitution of polysaccharides have shown this to be true. They state that "the average yield from the polysaccharide (*e.g.*, cellulose) to the hexose (*e.g.*, glucose) is thus 95.5 per cent. of the theoretical amount. Considering the standard of purity in which the mixed methylglucosides were isolated, there can be no further doubt that cotton cellulose is composed entirely of glucose residues."

Pringsheim (1912) observed cellobiose—a disaccharide which bears the same relation to cellulose that maltose does to starch and which spilts into two molecules of glucose (Fischer and Zemplen, 1904 and 1910), when acted upon by cellobiase—as an intermediate product of bacterial cellulose digestion. The exact mode of linking together of the two glucose residues in cellobiose was determined by Haworth and Hirst (1921). Groenewege (1920) showed that the bacterial digestion of cellulose is a hydrolytic process.

Cellulase is known to be produced by various moulds, soil fungi, pathogenic fungi, actinomycetes, soil and intestinal bacteria. Cellase is to be distinguished from cellulase in that it acts on the hemicelluloses and is not an endo-enzyme (Pringsheim, 1912).

Some rather divergent views have been expressed regarding the products of cellulose digestion; different investigators working with different cellulose-decomposing organisms have gotten different results. But according to Cross and Dorèe (1922) most of these differences may be accounted for. The typical result of the digestion of wood cellulose is about as follows: Cellobiose, glucose, acetic acid, lactic acid, butyric acid, alcohol, $CO_2$, $H_2$, $CH_4$, CO. Under excessive aëration alcohol and acetic acid are completely oxidized to $CO_2$ and $H_2O$. The gas liberated is never pure $CO_2$; there is either $H_2$ or $CH_4$, or a mixture of the two. Alcohol is never obtained without acetic acid. Waksman (1922)

is of the opinion that some soil bacteria cause $CH_4$ fermentation of cellulose and that others cause $H_2$ fermentation. McBeth (1913) thinks cellulose-fermenting organisms are not responsible for the production of gases. Gas, he thinks, is produced by contaminating organisms.

Abstracting Klason's remarkable researches on lignin, Cross and Doree (1922) state: "*Beta*-lignin ($C_{19}$ $H_{18}$ $O_9$, and that part of lignin precipitated by arylamine bases is designated as acrolein-lignin or *alpha*-lignin. The other part, which is not precipitated, appears to be a carboxyl group and is called carboxyl lignin or *beta*-lignin) must therefore be bound to cellulose and probably to *alpha*-lignin ($C_{22}H_{22}O_7$). Lignin cannot well be assumed as a secondary product derived from cellulose, but appears as a direct assimilation product of $CO_2$ and $H_2O$ or formaldehyde. Therefore the formation of lignin is a function of chlorophyll." Klason thinks lignin is possibly present in wood as a glucoside and that it may be built up from pentose.

## THE RELATION OF THE PROTOZOA TO THEIR HOST.

### *A.* Historical.

#### 1. *True Parasites.*

Most students of termites, and termite protozoa, have focused their attention on the morphology[1] and behavior of either the termites or the protozoa which they harbor; consequently, the interrelation of host and parasite has been very little investigated.

Grassi is really the only investigator who has not confined his attention almost exclusively to a study of either the termites or their intestinal protozoa. He has studied the termites and the protozoa, but has never carefully investigated their relationship. His work on the two groups of organisms, termites and termite protozoa, has been largely a study of two separate and distinct problems; namely, the morphology and systematics of the protozoa, and the physiology and behavior of the termites, with special emphasis on the underlying factors in caste production.

Grassi and Sandias (1893) think that the relative abundance of protozoa present in the various adult individuals in a termite

[1] The morphology of termite protozoa is a fascinating subject and occupied the writer's entire attention for a year before the present investigation began. He will publish in a later paper a description of several new forms.

colony is inversely proportional to the degree of gonad development of their host. According to these investigators, the protozoa, with a small amount of saliva, function in the development of soldiers and workers. Why the same thing (many protozoa and a small amount of saliva) functions in the production of two separate and distinct castes, they do not consider. They observed that the protozoa were scarce in the winged sexual forms, and usually totally absent in the neoteinic [1] individuals. When they found protozoa abundant in the neoteinic individuals, the gonad development was imperfect. They remarked that the developing neoteinic forms were fed largely on a salivary diet, which they thought killed the protozoa, though it is more probable that the protozoa died because their host no longer furnished them with wood for food. Then, according to Grassi and Sandias, it is impossible to say whether the maturation of the gonads is due solely to the change from a wood to a salivary diet or to the absence of protozoa. In this paper they reach no definite conclusion, though they do not believe the presence or absence of protozoa a sufficient cause to control gonad development, for they say: "It is a moot point whether the maturation of the generative organs is due solely to the saliva or to the absence of protozoa as well; but the latter, as my [2] previous remarks show, is not by itself a sufficient cause." Then in the next paragraph," I have frequently asked myself whether the protozoa have not an important digestive function, since the comminuted wood passes almost entirely through their bodies. It is probable, but not proved."

Brunelli (1905) followed up the idea of Grassi and Sandias, and states that in the queens of two genera harboring protozoa, *Kalotermes flavicollis* and *Reticulitermes lucifugus*, there is a correlated destruction of the oöcytes—a kind of indirect parasitic

[1] The term neoteinia was first introduced by Camerano (*Bull. Soc. Ent. Ital.*, 1885, pp. 89–94) to note the persistence during adult life of part or all of the characteristics normally peculiar to the immature, growing, or larval stages. Grassi and Sandias probably refer to the second and third form reproductive castes since it has been shown by Thompson (1920) that the jaw muscles of these forms degenerate to the extent that they cannot eat wood and must be fed on a fluid diet; and, of course, we should not expect them to harbor intestinal protozoa when they do not eat wood.

[2] Grassi wrote most of the paper. The part written by Sandias is placed between asterisks.

castration ("castration parasitaire"). Feutand (1912) says what Brunelli regards as signs of degeneration is in reality nothing more than an alteration of tissue brought about by the histological reagents.

Grassi, nineteen years later (1911), collaborating with Foa, is of the opinion that the protozoa do not aid their host in the digestion of food, and he points out that many wood feeding insects do not harbor protozoa. He states[1] that several workers (species not mentioned) of *Kalotermes*[2] were placed in small boxes with wood and incubated at about (*circa*) 35° C. It is interesting to note that none of the protozoa harbored by *Kalotermes schwarzi* Banks, from Miami Beach, Florida, are killed when incubated for three days at 35° C. How long Grassi's species of *Kalotermes* was incubated is not known, for he does not mention this in his paper. But he does state that the incubation killed off nearly all the protozoa, all of the large forms being killed off every time. These termites, he states, lived in full activity for several months.

Recently Jucci (1920 and 1921), in a preliminary paper reported before the Academy of Lincei, has announced the discovery of a particular diet which brings about caste production. But the carefully controlled breeding experiments and the numerous field observations of Thompson and Snyder (1919, 1920, etc.) make it highly probable that at least three of the castes, namely, the first, the second and the third form reproductive males and females, only give rise to fertile reproductive males and females like themselves and to infertile males and females of the soldier and worker castes.

### 2. *Commensals.*

If these entozoic protozoa benefit from their association with the termites, and by so doing do not harm or benefit their host, they are commensals. They may feed from the waste food-

[1] Per mio conto in questi ultimi anni ho cercato di decidere la questione in altro modo: se si tengono ad una temperatura di circa 35° C. scatolette contenenti legno pieno di *Calotermes*, i Protozoi muoiono, talvolta tutti, più spesso restano in vita soltanto le forme piccole; si hanno così delle colonie di *Calotermes* senza *Joenia* e *Mesojoenia*, e talvolta con molti individui anche del tutto privi di Protozoi. Io le tengo in vita prospera da parecchi mesi; perciò ritengo che i *Calotermes* possano digerire il legno anche senza gli speciali Protozoi (*Joenia* e *Mesojoenia*).

[2] Also spelled *Calotermes*

products and bacteria in the termite's intestine, or they may be food-robbers and feed from the same table as their host; in either case, they are commensals, provided the host's table always contains sufficient food for both host and parasite. Kofoid and Swezy (1919) are the principal exponents of the commensalistic relationship of termite protozoa. They remark: "One of the most curious and unique faunal associations to be found among the parasitic [1] protozoa is the group parasitic [1] or commensal in the intestinal tract of the social termites. . . . This distension is caused by the vast numbers of parasitic and commensal protozoans which fill the lumen of the intestine. When this is opened a thick milky fluid exudes. Under the lens this is found to be composed of great quantities of these small forms, thickly massed together, along with fragments of wood upon which the host, as well as some of the commensals, feeds."

### 3. *Symbionts.*

Imms (1919) in studying the termite *Archotermopsis wroughtoni* Desneux made sections of twelve worker-like females and found numerous protozoa and nearly full sized ova ready for fertilization, and in no instance did he observe even an indication of oöcyte degeneration. He also found well developed ovaries in five soldiers, whose hind-intestines were filled with protozoa. Imms suggests that the presence of protozoa may be correlated with the wood feeding habit of the termites. In the young larvæ, and in the queens and kings, which receive the fluid diet from the other members of the colony, he found, as a rule, no protozoa. In the older larvæ, soldiers, workers, and sometimes in the winged sexual forms,[2] protozoa are abundant and a wood diet is the rule.

[1] This term, loosely used, means any organism which lives in or on another organism.

[2] It is interesting here to note the statement of Snyder (1920), regarding one of his cross-breeding experiments. He says, "A large series of young second form female adults of *Reticulitermes flavipes*, which possibly may have been fertilized by second form males, were taken from a fairly small colony and placed with mature first form dealated males, which had not copulated, in small shallow cells in decayed wood sunken in moist sand in glass jars and tin boxes.

After a period of ten days to two weeks all these second form females had died, but the first form males were still living and were active; they evidently were prepared to forage for themselves, whereas the second form adults needed the care and nourishment usually afforded them by the workers. Possibly the jaw muscles

The wood, according to Imms, after being crushed by the mandibles and later by the gizzard, passes rapidly to the hind-gut without undergoing any chemical change. The pyloric valve prevents the readmission of wood particles into the mid-gut. To quote Imms: "On reaching the hind-intestine the wood is in a condition of minute fragments and particles, and the greater bulk of it gradually becomes taken up and absorbed by the numerous intestinal protozoa. Within the protoplasm of these organisms the woody material undergoes chemical changes, and, when ejected from the bodies of the protozoa, much of it is in a condition capable of being assimilated as food by the host termite. A significant fact is that lignous particles are not subjected to the action of the secretions of the mid-gut."

Imms further points out that, in his opinion, the experiments of Grassi and Foa (1911), where these authors claim to have kept *Kalotermes* (species not given, nor is the number of termites) alive and active for several months with all but a few of the intestinal protozoa killed off by incubation at about 35° C., has no value, for during incubation the termites very probably subsisted on the large amount of fecal matter [1] which had accumulated in the jars where they had been kept previous to incubation.

Imms, then, through an ingenious method of reasoning, reaches the conclusion that the protozoa are symbionts, a conception already reached by Buscalioni and Comes (1910), who claim, by means of various microchemical tests, to have shown that the wood is digested inside the bodies of the protozoa; and since the protozoa digest the wood, these authors conclude, that they are symbionts. [2] They state that *Trichonympha agilis*, harbored by *Reticulitermes lucifugus*, when treated with iodine dissolved in iodide of potassium, gives a characteristic glycogenic reaction in a region near the nucleus, and that this reacting region is sharply

in these young second form adults had begun to degenerate through disuse in masticating wood. It is believed that second form males—like the females—would have succumbed, without the workers." First form adults, so far as my experience goes, harbor protozoa, but the second forms do not. This probably explains the inability of the second form adults to live without workers, that is in the event the jaw muscles permitted them to eat wood. Of course the protozoa are lost, possibly, because the host does not eat wood. I have experiments started which I hope will determine this point.

[1] The fecal matter contains sugars and possibly other foods.

[2] A discussion of this may be found on pages 193–4.

defined from the rest of the body. Cutler (1921) could not locate a definite glycogen reacting area in *Pseudotrichonympha pristina*, harbored by *Archotermopsis wroughtoni*, but, on the contrary, found that the glycogenic reaction was diffused through the entire organism. Grassi and Foa (1911) state that they in part attempted to verify the work of Buscalioni and Comes but that their results did not justify the drawing of any conclusions.

Dobell (1921) says: "When the association benefits both parties, the condition is one of symbiosis—a not very frequent state in nature. An example is afforded by some of the flagellates living in termites. In return for the food and lodging which the termite gives to the flagellate, the latter helps the former digest its own food." Regarding this statement Dobell (1922)[1] says: "It is merely a general statement of my own personal view. It is not based on any particular experiments which I have made, but is an inference from what I myself have observed and others have recorded. For many reasons I consider it probable that the flagellates of termites aid their host in digesting the wood on which they feed. The observations of Buscalioni and Comes (1910) and of Imms (1919) especially seem to me to justify such a conclusion.

"However I am quite ready to admit that the matter has not been definitely settled beyond all question. In fact I recently discussed the problem with Dr. Koidzumi and we planned several experiments to determine the question. . . ."

### *B.* Original Observations.

At best some of the experiments of Buscalioni and Comes (1910) were conducted in a rather crude manner and, in addition to this, unwarranted conclusions were drawn due, often, to misinterpretation of the results obtained. The only conclusion which may be drawn from the results of their experiments is, that the protozoa digest the wood which they take into their bodies. But the fact that the protozoa digest the wood in no way hinders the termite from doing the same thing. Many animals ingest more food than they can use. The protozoa may feed from the wood particles in the termite's intestine, as one would naturally expect them to do, since nutrition with most of them appears to be holozoic.

[1] Personal communication.

In order to establish the fact that the protozoa are symbionts, rather than commensals (feeding from the same table as their host), it must be shown that they actually aid their host in some way by doing something for the host which the host itself cannot do. The experiments of Buscalioni and Comes do not in any way fulfill this condition. They have only shown that the protozoa digest wood particles. The ability or inability of the termites to do the same thing was not studied. That the termites aid the protozoa, by furnishing them food and lodging, is obviously demonstrated by the fact that the protozoa, so far as known, cannot live outside of termites. But we cannot call the protozoa symbionts, until their ability to digest wood has been shown to be a necessary aid to the termite.

Realizing the difficulty of determining the relation of the protozoa to the termites so long as the two were associated together, several experiments were begun, with the hope of effecting a method for removing the protozoa from their host, and at the same time leave the host uninjured. It is needless to say that hundreds of experiments—some of which are given in Table I.—met with failure, for the host was either killed or severely injured in an effort to remove the protozoa from it.

When wood, previously soaked in a 5 per cent. aqueous solution of either sodium, potassium or calcium chloride, was fed to a colony of *Reticulitermes flavipes* (elsewhere in this paper—unless it is otherwise evident—when the words "termite," "termites" and "host" are used they refer particularly to this termite) harboring protozoa, within four to five days an occasional termite, free, or almost free, of protozoa was found, when many individuals from the colony were examined. The number free of protozoa increased as time progressed but, before all the termites in the colony had lost their protozoa, it was quite evident that the termites themselves were not normal. They had in some way been affected; whether by a direct action of the chemicals or by a gradual loss of the protozoa, it is impossible to say from the data available.

About this time a much more promising method for removing the protozoa was evolved; namely, the incubation method, and the experiments employing the use of salts, etc. (see Table I.), to remove the protozoa, were discontinued. It might be well to

mention here that, because of the success of the incubation method, many of the other attempts to free termites of their intestinal protozoa were, perhaps, not given a fair trial. If different dilutions of some of the chemicals had been used, the results, possibly, would have been more promising. Had X-ray and ultra-violet light been used more extensively, excellent results might have been obtained.

It was found that all—not one was left alive—of the protozoa could be removed from the intestine of *Reticulitermes flavipes* by incubating them for 24 hours at 36° C. This was a much more rapid method than the feeding of salts, as described above, where several days are required to remove the protozoa from all the individuals in a colony and, besides, the termites after incubation seemed to be perfectly normal in every way. All other attempts to remove the protozoa were now abandoned in favor of the incubation method, which may be briefly described as follows: Sometimes stumps and large pieces of wood, containing thousands of termites, were brought to the laboratory without molesting or disturbing the colony. The stumps and large pieces of wood were placed in glass jars and incubated for 24 hours at 36° C. At the end of the incubation period the jars were taken from the incubators and, after many termites from each jar had been carefully examined to be certain that the protozoa had all been killed, were left at room temperature, which was usually about 20°C. Sometimes the stumps and logs were split before they were brought to the laboratory and the termites were taken from them and placed, with a good quantity of wood, in glass jars with metal tops. These jars were placed in the incubator as soon as they reached the laboratory. In other experiments, where termites were counted, small shell vials with cork stoppers were used, and from 10–100 termites were placed in each vial, and incubated in the same way that those in the large jars were. Several vials of termites without wood, and several with pure cellulose [1] (Whatman's filter paper) only, were incubated. Other experiments in incubation were carried out with results as shown in Table I. One very significant fact brought out by these ex-

[1] This paper was made by W. & R. Balston, Ltd., and contains .0004 grams of ash per circle of 55 mm. diameter. When "pure cellulose" occurs in this paper it refers to this grade of the genuine Whatman filter paper.

periments is that the thermal death point of the protozoa is 3–4° C. lower than that of the insect host.[1]

After incubation the termites were closely observed to note if they at any time became abnormal; to prevent the growth of moulds in the jars, for moulds are very destructive to termites; to be certain that the jars contained neither too much nor too little moisture—a thing that can only be learned from long experience in keeping large numbers of termites in captivity and an intimate knowledge of their habits in nature. Every known precaution was used to keep the termites during incubation in exactly the same environment, except temperature, as the unincubated controls. After incubation the incubated and the unincubated termites were kept under identical conditions, as regards food, temperature, moisture, light, etc.

The termites become abnormal in from five to fifteen days after they have been removed from the incubator. They are less active and their abdomens, if carefully examined, may be seen to be smaller and slightly flattened. The length of time required for abnormalities to appear depends no the kind of wood fed after incubation, the more decayed the wood the longer it is before any abnormalities appear. In two to five days after this first symptom is noticed the abdomen becomes still more flattened. Soon it is very much smaller and almost flat, and two or three days later the animal can scarcely be made to move at all, for only a modicum of vitality remains. Death occurs, on the averrage, from 10–20 days after incubation. It occurs, as shown in Table II., in some instances in less than ten days and in a few cases after twenty days, the longest being 27 days. In the less-decayed wood, as mentioned above, death occurred early, and in the most decayed, it occurred late. But why did it occur at all? Why did the incubated termites die?

The death of the incubated termites—sterile, uninfected, uninfested and defaunated, so far as protozoa are concerned—may be due to the change in temperature, *i.e.*, the incubation *per se*; it may be due to the killing off of all the intestinal protozoa; or it may be due to the killing of intestinal bacteria and

[1] *Termopsis* sp. from Ashland, Oregon, is killed at a lower temperature. It is killed in less than 24 hours at a temperature of 35.5 degrees C. Work on the T. D. P. of termites from various parts of the world is now in progress and the results will be published in a later paper.

fungi, which, possibly, in some way aid their host in the digestion of wood and pure cellulose.

## TABLE I.

METHODS AND RESULTS OF VARIOUS ATTEMPTS TO FREE TERMITES OF THEIR INTESTINAL PROTOZOA.

| Method. | Number of Experiments Carried Out. | Approximate Number of Termites Used in Each Experiment. | Results. |
|---|---|---|---|
| Wood,[1] 5% NaCl | 30 | 500 | Kills protozoa in 10–15 days, but host is almost dead by this time |
| Wood,[1] 5% $CaCl_2$ | 10 | 100 | Same result as with NaCl |
| Wood,[1] 5% KCl | 20 | 500 | Same result as with NaCl |
| Wood,[1] $HgCl_2$ | 20 | 500 | Host killed in Dil. of 1/15,000 |
| Wood,[1] $CuSO_4$ | 5 | 100 | Killed host |
| Quinine sulphate | 2 | 100 | Killed host |
| Cinchona bark | 2 | 100 | Killed host |
| Gentian violet | 15 | 100 | Dil. 1/100,000, host and protozoa unaffected |
| Acid fuchsin | 10 | 100 | Dil. 1/100,000, host and protozoa unaffected |
| Anhydrous $CaCl_2$[2] | 6 | 500 | Many protozoa killed, host injured |
| Conc. $H_2SO_4$[2] | 5 | 200 | Many protozoa killed, host injured |
| Hornet's nest | 5 | 2,000 | Host and protozoa unaffected in 2 mos. |
| Humus | 10 | 10,000 | Host and protozoa unaffected in 3 mos. |
| Soil | 5 | 1,000 | Host and protozoa unaffected in 2 mos. |
| Soap | 3 | 100 | Host killed |
| Leather | 3 | 50 | Host and protozoa unaffected in 3 weeks |
| Low temperature | 5 | 200 | 2–3° C., no effect in 3 weeks |
| Starvation | 20 | 1,000 | Many protozoa killed, host injured |
| Ether fumes | 4 | 100 | 1 min. no effect on protozoa or host |
| Fumes of $C_2H_5OH$ | 4 | 200 | 2 min. no effect on protozoa or host |
| Fumes of $CHCl_3$ | 2 | 50 | 1/2 min. no effect on protozoa or host |
| U.V. Light, 10 in. | 10 | 500 | 15 min. no effect on protozoa or host |
| X-ray, 2 skin dos. | 10 | 500 | No effect on protozoa or host |
| Inc[4] 35°.5 C. 24 hrs. | 15 | 5,000 | Few protozoa killed, host uninjured |
| Inc[4] 36° C. 24 hrs. | 4 | 100,000 | All protozoa killed, host uninjured |
| Inc[4] 42° C. 10 min. | 10 | 500 | Few protozoa killed, host uninjured |
| Inc[4] 43° C. 10 min. | 10 | 500 | All protozoa killed, host uninjured |
| Inc[4] 44° C. 10 min. | 10 | 500 | Host not killed |
| Inc[4] 45° C. 10 min. | 10 | 500 | Host not killed |
| Inc[4] 46° C. 10 min. | 10 | 500 | Host not killed |
| Inc[4] 47° C. 10 min. | 10 | 500 | Host nearly all killed |
| Inc[4] 48° C. 10 min. | 10 | 500 | Host all killed. 48° C. is T. D. P. of host |

[1] Wood moistened with 5 per cent. Aq. solution of NaCl, KCl, $CaCl_2$ and with many dilutions of $CuSO_4$ and $HgCl_2$

[2] These chemicals were used in dehydration experiments.

[3] More than 2,000 experiments were carried out.

[4] Incubated.

*Kalotermes schwarzi*, *K. jouteli* and *Prorhinotermes simplex* when incubated for 24 hours at 36° C. are defaunated, but enough material was not available to carry out extensive experiments on these forms as was done with *Reticulitermes flavipes*.

TABLE II.

SHOWING THE LENGTH OF LIFE OF INCUBATED AND NON INCUBATED TERMITES WHEN FED THEIR NORMAL DIET OF WOOD. 30 EXPERIMENTS SELECTED AT RANDOM FROM A SERIES OF 500.

| No. of Experiment | Treatment. | Approximate No. of Termites Used in Each Experiment. | Length of Life in Days. | Remarks. |
|---|---|---|---|---|
| 1*a*7 | Incubated | 4,000 | 14 | |
| 2*a*7 | Unincubated | 2,000 | 60 | Jar became too dry due to neglect |
| 11*a*15 | Incubated | 500 | 17 | |
| 7*a*15 | Unincubated | 1,200 | In.[1] | Alive now, 9 months later |
| 12*a*15 | Incubated | 400 | 15 | |
| 8*a*15 | Unincubated | 1,000 | In.[1] | Alive now, 9 months later |
| 13*a*17 | Incubated | 300 | 20 | |
| 14*a*17 | Incubated | 500 | 20 | |
| 15*a*17 | Incubated | 400 | 21 | |
| 1*a*17 | Unincubated | 700 | In.[1] | Used in other expts. after 6 months |
| 2*a*17 | Unincubated | 2,000 | In.[1] | Alive now, 8 months later |
| 2*a*27 | Incubated | 300 | 18 | |
| 2*b*27 | Incubated | 200 | 21 | |
| 3*b*27 | Unincubated | 700 | In.[1] | Alive now, 5 months later |
| 3*a*27 | Unincubated | 500 | In.[1] | Alive now, 5 months later |
| 1*d*27 | Incubated | 400 | 7 | Mould caused early death |
| 2*c*27 | Incubated | 600 | 15 | |
| 1*a*28 | Incubated | 200 | 10 | |
| 2*a*28 | Incubated | 100 | 14 | |
| 3*a*28 | Unincubated | 200 | 45 | Used in other experiments |
| 1*a*31 | Unincubated | 1,500 | In.[1] | Alive now, 4 months later |
| 2*b*31 | Incubated | 500 | 12 | |
| 3*b*31 | Incubated | 700 | 18 | |
| 2*a*31 | Incubated | 1,000 | 8 | Wood decayed very little |
| 4*a*31 | Incubated | 2,000 | 10 | Wood decayed very little |
| 1*c*32 | Unincubated | 2,000 | In.[1] | Used in other expts. after 3 months |
| 2*c*32 | Incubated | 1,500 | 27 | Wood very much decayed |
| 1*d*32 | Unincubated | 3,000 | In.[1] | Used in other expts. after 3 months |
| 2*d*32 | Incubated | 2,000 | 20 | |
| 1*f*33 | Unincubated | 1,000 | In.[1] | Alive now, 4 months later |

To be certain there was no error in the experiments which had been done up to this time, more than five hundred jars of termites were collected; some were incubated and some were not incubated, as shown in Table II. No doubt 100,000 termites

[1] Indefinitely.

were used in these experiments and, in every case, death occurred, as in the preliminary experiments, within 10–20 days on the average after incubation. In no case did the unincubated and faunated controls die. In fact, they are still alive now, more than six months since many of the experiments were completed.

How may the cause of the incubated and defaunated (with the protozoan fauna removed) termites' death be determined? Is death a direct reslt of the incubation? That is, if the termites did not harbor intestinal protozoa which are killed off by the incubation, would death result in 10–20 days? In other words, is the death of the protozoa harbored by the termite in any way connected with or responsible for the death of their host; or is it an entirely independent phenomenon having nothing at all to do with the termite's death. In an effort to answer these questions many experiments were carried out, but only those experiments whose results throw some light on the questions involved will be mentioned and discussed.

Insects, like the cockroach, rather closely related to the termites morphologically, and many wood-boring beetles, rather closely related to termites in habits, were incubated at the same temperature for the same length of time, and for a longer time, as were the termites. These were carefully observed for two months after incubation and no abnormalities were ever observed in any of them. It seems, then, that this temperature is not detrimental to these insects. But the fact that a cockroach or a wood-boring beetle is not killed by the incubation method employed in killing the protozoa of termites, does not show that the termites themselves were not killed directly by incubation, because these insects do not harbor a vast multitude of intestinal protozoa that are suddenly killed off, as do the termites; and besides, they are, after all, quite different in some respects from termites.

*Nasutitermes morio* Latreille, from Porto Rico, one of the Termitidæ, and, of course, a termite which does not harbor intestinal protozoa, was procured for experimental purposes, the idea being that if this termite could withstand the same incubation temperature that *Reticulitermes flavipes* had been subjected to, and receive no ill effects therefrom, as evidenced by its ability to live indefinitely after incubation, the probability that *Reticulitermes*

*flavipes* was not killed directly by incubation was very much augmented. Then, some cause other than the effect of the increased temperature on the cells of the termite might be expected to be responsible for the termite's death. But *Nasutitermes morio* could not be kept in closed jars for a longer period than 5–10 days before death occured, regardless of whether they had been incubated or not. *Amitermes* sp. from Uvalde, Texas, another genus of the Termitidæ, was obtained, but it also could not be kept in closed jars. Other genera of the Termitidæ, termites not harboring protozoa in nature so far as known, which might possibly be kept in closed jars—though nothing at all is known regarding this—were not obtainable. It then became necessary to answer in another way the question, does the heating kill the termite directly?

An effort was made to keep the incubated and defaunated termites alive by feeding them substances other than wood, their normal diet. Dextrose, peptone and starch, separately and altogether, were fed them; and it was evident, from the results (see Table III.) obtained by the feeding of these substances, particularly dextrose, that the lives of the incubated and defaunated termites had been prolonged.

But before these experiments had progressed very far a much better method of prolonging the lives of the defaunated termites was discovered, and no more experiments employing dextrose, starch and peptone were carried out. They were placed in glass jars and were fed a diet of fresh humus, which seemed to be a very satisfactory food, as the results tabulated in Table III. show. When they were given humus before any of the characteristic symptoms of wood-fed defaunated termites had appeared, no such symptoms ever appeared. If these symptoms had appeared before humus was fed, the termites after being fed humus for three or four days became much more active, and within six to seven days they were normal again. Even when all the individuals in a jar of defaunated termites were almost dead, due to being fed on wood, many of the almost lifeless ones could be brought back to normal by feeding them humus. The humus fed defaunated termites appeared to be perfectly normal in every way long after all of their wood fed defaunated controls were dead. One hundred jars of defaunated termites were used in the

## TABLE III.

SHOWING THE RESULTS OF ATTEMPTS TO PROLONG THE LIVES OF INCUBATED AND DEFAUNATED TERMITES BY FEEDING THEM VARIOUS SUBSTANCES. 27 EXPERIMENTS SELECTED AT RANDOM FROM A SERIES OF 100.

| No. of Experiment. | Substance. | Approximate No. of Termites Used in Each Experiment. | Length of Life in Days. | Remarks. |
|---|---|---|---|---|
| 2a15 | Dextrose | 200 | 30 | |
| 3a15 | Dextrose | 200 | 34 | |
| 4a15 | Dextrose | 200 | 30 | |
| 5a15 | Peptone | 200 | 4 | |
| 10a15 | Dextrine | 100 | 39 | |
| 7a15 | Dex. & Pep.[1] | 100 | 15 | |
| 9a15 | Dex. & Pep.[1] | 100 | 43 | |
| 3a19 | Dextrose | 150 | 30 | |
| 4a19 | Dextrine | 200 | 51 | |
| 4f33 | Humus | 1,000 | 125 | Experiment discontinued[3] |
| 4g33 | Humus | 1,000 | 43 | Allowed to become too dry |
| 2g33 | Humus | 500 | 125 | Experiment discontinued [3] |
| 12a35 | Humus | 1,000 | [illegible] | Experiment discontinued [3] |
| 13a35 | Humus | 1,000 | [illegible] | Experiment discontinued [3] |
| 22a35 | Humus | 1,000 | [illegible] | Experiment discontinued [3] |
| 23a35 | Humus | 1,000 | [illegible] | Experiment discontinued [3] |
| 36a35 | Humus | 1,000 | 40 | Changed to a wood diet [4] |
| 20a35 | Humus | 1,000 | 45 | Changed to a wood diet [4] |
| 19a35 | Humus | 1,000 | 20 | Changed to a wood diet [4] |
| 9a63 | Soil | 1,000 | 50 | Experiment not done with care |
| 10a61 | Soil | 200 | 45 | Experiment not done with care |
| 17a35a | Int. ct.[2] | 50 | 40 | Experiment discontinued [3] |
| 17a35b | Int. ct.[2] | 50 | 35 | Experiment discontinued [3] |
| 17a35c | Int. ct.[2] | 50 | 34 | Experiment discontinued [3] |
| 1a45 | F. D. C.[5] | 50 | 60 | Experiment discontinued [3] |
| 2a45 | F. D. C.[5] | 50 | 60 | Experiment discontinued [3] |
| 4a45 | F. D. C.[5] | 50 | 60 | Experiment discontinued [3] |

humus feeding experiments, and from ten to more than a thousand termites were placed in each jar. It does not seem feasible or necessary to state when each of the one hundred jars were collected and incubated and just what happened to each of them, since the results of each experiment are similar, as Table III. shows.

[1] Dextrose and peptone.

[2] Intestinal contents of wood-boring insect larvæ.

[3] No evident signs of death at this time.

[4] Death resulted in 10–20 days later.

[5] Fungus digested cellulose.

*(To be continued)*

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# BIOLOGICAL BULLETIN

## THE PHYSIOLOGICAL AND SYMBIOTIC RELATIONSHIPS BETWEEN THE INTESTINAL PROTOZOA OF TERMITES AND THEIR HOST, WITH SPECIAL REFERENCE TO *RETICULITERMES FLAVIPES* KOLLAR.*

L. R. CLEVELAND.

(*Continued*)

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This is the second of a series of papers on this subject. The first paper of the series, "Correlation between the Food and Morphology of Termites and the Presence of Intestinal Protozoa," deals with the more general aspects of the subject, and will appear in the July (1923) number of the *American Journal of Hygiene.*

After the incubated and defaunated termites had been fed humus and kept alive for a longer period of time than they would have lived had they been fed wood, as shown by the death of all the wood fed defaunated controls; from time to time transfers back to a wood diet were made, with the result, in each case, of death in ten to twenty days, the usual length of life of wood-fed defaunated termites. The last of the transfers back to wood was made after the defaunated termites had been kept alive, active, and apparently normal in every respect, on a humus diet for three months. Death resulted, as was expected from the previous experiments, and two weeks after all the defaunated termites which had been transferred from the humus to the wood diet were dead, the humus-fed defaunated termites were alive and active, at which time the experiments were discontinued. That progressive development took place is evidenced by the fact that molting occurred in the humus-fed defaunated termites three months after all the protozoa had been removed by incubation. Deeply pigmented first form reproductive adults were formed in several of the jars during December and January.

From these experiments it is obvious that the humus which was fed to the defaunated termites is potent to prolong their life four months—and probably indefinitely—beyond the death point of such termites when fed a diet of wood. The unincubated controls, which had been fed wood and kept in the laboratory in the same way as the incubated and defaunated termites being fed wood and the incubated and defaunated termites being fed humus, were alive and active at the time the experiments were discontinued. Now, since the incubated and defaunated termites live indefinitely when fed humus, but die in two to three weeks when fed wood, and the unincubated and faunated termites live indefinitely when fed wood, it is evident that the death of the incubated and defaunated termites is not due directly to the incubation temperature, but to an acquired inability to make use of wood as food. In other words, the termites after incubation and the removal of the protozoa, are no longer able to maintain themselves on their normal diet of wood, and death results. The incubation has in some way incapacitated them as wood users. They feed on the wood, as may easily be determined by watching them or by examining their intestinal contents, but evidently are

unable, for some reason, to utilize it—perhaps digest it, since humus, which is decomposed or digested wood, will keep them alive indefinitely.

When the protozoa are treated with a hydrocholric acid and phloroglucinol solution a distinct pink color appears inside the bodies of the wood ingesting protozoa. Imms (1919) used this reaction to prove that the particles which he saw inside the bodies of the protozoa were wood particles—"ligneous particles." Oshima (1919) placed phloroglucinol and hydrochloric acid on the termite intestinal contents and interpreted the macroscopic pink color reaction, which appeared immediately after the solution was placed on the intestinal contents, as a positive test for lignin and, from this experiment, he drew the conclusion that lignin passes through the termite's (*Coptotermes formosanus*) alimentary canal unacted upon. Buscalioni and Comes (1910) interpreted the phloroglucinol reaction as indicative of the presence of wood sugars in the bodies of the protozoa, which sugars, these investigators claimed, the protozoa had by enzymatic action elaborated from the ingested lignocellulose particles. According to Sherrard (1922) no wood sugars can be obtained from wood except by extracting the cellulose. Mannan, for instance, very probably exists in the form of manno-cellulose. In every case the quantity of cellulose removed corresponds to the quantity of sugar produced. In other words, the amount of sugar obtained is proportional to the cellulose extracted. Ritter and Fleck (1922), in making an anlyasis of some American woods, obtained a yield (a mean of four samples) of 0.82 per cent. of pentosan content from western yellow pine (*Pinus ponderosa*) extracted cellulose and a yield of 22.82 per cent. from tanbark oak (*Quercus densiflora*) extracted cellulose.

Aldopentoses, such as *l*-xylose and *l*-arabinose, when warmed with phloroglucinol and hydrochloric acid, give a cherry-red color to the solution. But this color reaction does not take place in the cold. Crocker (1921) states: "No case is known where materials conceivably present in wood, other than aldehydes, can react with the phloroglucinol reagent, in the cold, to produce a red color." Crocker reaches this conclusion after having tested a large number of chemically pure compounds. Klason [1] thinks

[1] Svensk Pappers-Tid; 23 (1920), p. 70.

the principal color-forming aldehyde of wood is coniferyl aldehyde. Cross and Dorée (1922) conclude: "We have thereform a direct confirmation of the view that the color reactions of lignocellulose are those of derivatives and associated furfural products."

When tested with aniline acetate most of the wood ingesting protozoa are colored yellow, which coloration is probably brought about by the presence of methylfurfuraldehyde (Cunningham and Dorée 1914). When tested with the same chemical, at the same time, a small percentage of the wood ingesting protozoa and many of the wood particles free in the termite's (*Termopsis*) intestine are colored pink, which coloration is probably brought about by the presence of a trace of furfuraldehyde, for Cross and Dorée (1922) state that "the aniline acetate test differentiates the aldehydes." Wood, before being ingested by the termites gives the same pink color reaction to phloroglucinol and hydrochloric acid that appears inside the bodies of the protozoa. Also, aniline acetate reacts with uningested wood in the same way that it does with the wood particles inside the bodies of the wood ingesting protozoa and the wood particles free in the intestine.

Now, what does the phloroglucinol reaction show? What is its value? It may show that the reacting substance (the substance producing the coloration) is a derivative of lignocellulose or a substance associated with lignocellulose. If the reacting substance is a derivative of lignocellulose, the lignocellulose bond or linkage has perhaps been broken, and it is highly probable that the protozoa are responsible for the breaking of it. Not many of the wood particles free in the intestine exhibit this reaction, and it is possible, of course, that the particles which do react have been inside the bodies of the protozoa. If the reacting substance is an aldopentose, as Buscalioni and Comes (1910) thought it was, it may be that the linkage or bond between aldopentoses and cellulose (Sherrard 1922) has been broken and, if so, it is highly probable that the protozoa have broken it. But, since the wood, before being ingested by the termites and reingested by the protozoa, gives the same color reaction that it does after ingestion, it is perhaps best to suspend judgment until more is known regarding the specificity of the phloroglucinol reaction.

However, the clear-cut, definite, and unmistakable glycogenic

reaction which appears inside the bodies of the wood-ingesting protozoa when treated with iodine in a solution of potassium iodide, is, perhaps, sufficient evidence to show that the protozoa exert a digestive action on the wood particles which they take into their bodies, for after the host has been fed cellulose (Whatman's filter paper. *Vide infra*) for three months glycogen is still present in the bodies of the protozoa and it cannot be demonstrated now, or at any time, in any of the cells of the host. The cellulose is probably split into cellobiose, the cellobiose into glucose, from which the glycogen is formed. The fact that no glycogen is present in the intestinal protozoa which do not ingest the wood particles or the cellulose particles, nor in the cells of the host, indicates strongly that the glycogen is formed inside the bodies of the protozoa that do ingest the wood particles or cellulose particles and that these protozoa are responsible for its formation. The intestine of the host is slightly alkaline (pH 7.2) which would make acid hydrolysis impossible. The cellulose, then, must be acted upon by enzymes.

It has not been possible to demonstrate the presence of fats in the bodies of the protozoa, though the presence of fats in the intestinal and other cells of the host may be easily demonstrated. Those termites which receive a fluid diet from other members of the colony accumulate an abundance of fat.

It is highly possible that the protozoa are dependent on their host for proteins, since they live much longer on a cellulose-inorganic culture medium to which proteins have been added.

In studying many of the termites of Japan, Oshima (1919) came to the conclusion that a preference was exercised in favor of those woods containing a high percentage of cellulose. He is of the opinion that cellulose is the only constituent of wood which termites use as food. This opinion is based on the following experiment which he carried out. Camphor wood was analyzed and fed to *Coptotermes formosanus;* the nest which was constructed after the wood had been eaten was analized, and it was noted that the chief difference between the camphor wood and the nest was in the cellulose-lignin ratio. The wood contained 48 per cent. of cellulose and 20 per cent. of lignin ($C_{40}H_{44}O_{15}$ Beckmann, 1921) while the nest contained 15 per cent. cellulose and 57 per cent. lignin. The method by which these percentages were ob-

tained is not given, and for this reason we have no way of judging their accuracy. Regarding this experiment Oshima remarks: "It is quite obvious that the amount of cellulose is the main difference between the constituents of camphor wood and those of the nest. As there occurs no decrease of noncellulose, it is clear that cellulose has been taken as the food when camphor wood passes through the alimentary canal; and noncellulose, that is lignin, which is produced as a decomposed material of lignocellulose by special function of the alimentary canal, is discharged as the building material of the nest."

Obviously, Oshima's data do not warrant the conclusions which he draws from them, for it is impossible, without doing a quantitative experiment, to establish the fact that lignin is not used as food and that cellulose is, since both substances appear in the wood and in the nest. Oshima completely disregards—purposely or ignorantly it is impossible to say—all substances which are present in the wood except cellulose and lignin. The fact that lignin is present in the feces (and the test used is not a test for lignin at all) does not prove that it is not used by the termite as food, for cellulose is also present in the fecal material and has to pass through the alimentary canal many times before it is used up. Perhaps it is all never converted into cellobiose and glucose. Then, it has not been shown that termites do not use lignin as food; nor has it been shown that they do.

Oshima (1919) fed cotton wool to *Coptotermes formosanus* and claimed that these termites lived more actively on this substance than when fed softwoods, the reason being, so he thought, that the softwoods have a lower percentage of cellulose. But the softwoods do not have a lower percentage of cellulose—at least those that have been anlayzed do not have. However, hardwoods average about 100 per cent. higher in pentosan content than softwoods. But Oshima's experiment has no value whatever, since the number of termites used and the length of life, when given a cellulose diet (Oshima's diet of cotton [1]), is not

[1] Purified cotton yields an ash ranging from 0.10 to 0.50 per cent., while raw cotton contains about 1 per cent. of mineral matter. Raw cotton contains about 90 per cent. cellulose and absorbent cotton 99 per cent. There is about 1 per cent. of pentosan in purified cotton. Most any filter paper, then, is a better cellulose diet than cotton in any form. Some filters contain an extremely small percentage of ash. Of course it cannot be said that the cellulose of filter paper is "normal"

stated. Many observers have noted that termites attack books, pasteboard, cloth, and wood pulp, but their ability to maintain themselves on these substances has not been investigated. Thinking it possible that termites in large numbers, when fed cotton,[1] do not maintain themselves solely on the substances present in the cotton, but continue to pass the wood, which they have previously eaten before being changed to a diet of cotton, through their bodies, digesting a portion of it during each passage, and thus giving the false impression of living on either the cotton or the filter paper, when in reality they were living on the wood particles which might still be present in their feces, I carried out the following experiment: One hunderd termites were placed in 100 vials, *i.e.*, one termite to each vial, and nothing but pure cellulose (Whatman's filter paper) was fed them. Many of these termites led an active existence and continued to harbor protozoa until the experiments were discontinued at the end of four months. Just how long termites can maintain themselves on a cellulose diet is not known, but is being investigated by the writer at the present time.

Another experiment, which was first done by accident, seems to throw considerable light on the direct cause of the death of the incubated and defaunated termites. Many of the defaunated termites were being fed cellulose and a strong cellulose-decomposing fungus accidently got into some of the vials, and it was noticed that the termites in the vials containing the cellulose-digesting fungus did not die within two to three weeks. Some of them lived in the vials with the fungus for more than three months, but in the vials containing some of the same defaunated termites and cellulose which was not digested by a fungus, death occurred within two to three weeks. When this accidental and preliminary experiment was repeated (see Table III.) the same results were obtained. It seems quite evident, then, that incubated and defaunated termites cannot digest cellulose.

cellulose. Cotton is really the only form of normal cellulose we have. Mayood and Cable (1922) have made a careful study of the cellulose of cotton and that of wood and conclude that the two are very similar, if not identical. The two, according to these investigators, are as much alike as the celluloses obtained from the same wood by acid cooking and by alkaline cooking. Until more is known regarding the nature of cellulose in various substances we may continue the usage of calling cotton "normal" cellulose.

Now, since the incubated and defaunated termites remain alive and active indefinitely (See Table III.) when fed humus—which is wood (chiefly cellulose) digested or decomposed by bacteria, fungi, actinomycetes—and digested cellulose, but die when fed wood or when fed cellulose, it seems very probable that death is due to an inability to digest wood. Futhermore, it has been shown that no sugars can be gotten from wood except by first extracting the cellulose, which probably means that the sugars exist in combination with the cellulose. Then, we may reasonably conclude that the inability on the part of the defaunated termites to digest wood is due, perhaps, to the disappearance of cellulase, brought about, in some way, by the incubation and removal of the protozoa.

If cellulase is no longer present in termites after incubation—and it does not seem to be—what is responsible for its disappearance? In order to answer this question the two factors involved in bringing about the disapparance of cellulase, incubation and the removal of the protozoa, must be separated. Reinfection of the incubated and defaunated termites with protozoa seemed to be the principal key with which this puzzling question might be unlocked, but unfortunately for several months it was impossible to replace the protozoa in the termites, once they had been thoroughly removed. Finally, a rapid and easy method of reinfection was developed. Since the reinfection experiments are the most important ones in the paper, perhaps, one of the seven which were carried out, should be given in detail just as done and recorded in my note book. The other six are very similar to the one recorded here.

Ten large jars of termites were collected and were numbered 1–10*p*45. Numbers 1–7*p*45 were incubated at 36° C. from 3:30 P.M., 10/21/22 to 3:30 P.M., 10/22/22. Numbers 8, 9 and 10*p*45 were not incubated and were left at room temperature (about 20° C.). Jars 1–7*p*45 were carefully examined on 10/23/22 and the termites in them were found to harbor no protozoa. In making this examination twelve individuals (workers), taken at random from each jar, were carefully dissected and their entire intestinal content painstakingly examined under the microscope for protozoa. If no protozoa were found, the colony was labeled "defaunated termites." Jar 6*p*45 was used as follows on 9/24/22,

the day after incubation: By means of a sieve the termites were separated from all pieces of wood and clumps of excrement larger than themselves. Then they were placed in beakers, and a piece of moist filter paper was placed in each beaker so that one corner just touched the bottom. The termites would leave the small pieces of wood and excrement and crawl up the filter paper, and when a large number of them were on the filter paper they were shaken from it and transferred to several small beakers. By this method the termites were entirely freed from all particles of wood and excrement. Now they were taken, one at a time, from the small beakers and placed in watch glasses. Here the right antenna was cut off close up to the head, and one by one, after the right antenna had been removed, they were placed in shell vials until ten individuals were in each vial. In this experiment 14 vials with 10 defaunated termites each were used. These were numbered 1–14*p*49. Some unincubated and faunated termites were taken from jar 9*p*45, collected at the same time that 6*p*45 was and taken from the same colony of termites in nature. These were freed from wood and excrement in the same way that the defaunated ones were. Then 10 unincubated and faunated termites were placed with 10 incubated and defaunated termites in vials 1–11*p*49; in vials 12, 13, and 14*p*49 fifty unincubated termites were placed. Workers only were used in all the experiments. Whatman's filter paper, moistened with distilled water, was put in each vial; the vials were tightly corked and left at room temperature in the dark. Results: 10/24/22 at 5 P.M. three termites from vial 8*p*49 were examined. Two contained no protozoa and one contained a few. 10/27/22 all of the individuals—those previously defaunated and those from which no protozoa had been removed by incubation—in 11*p*49 were examined. Two were dead, 12 harbored protozoa, and 6 harbored no protozoa. Three of the 12 that harbored protozoa had the right antenna cut off. This was a clear-cut case of refaunation (replacing of the protozoa in the intestine after they had been removed by incubation). The number of protozoa present in these refaunated or reinfected individuals was about half normal.

On 12/29/22 six vials were examined with results as follows:

Number 3*p*49 contained 16 termites, 6 with no right antenna.
" 4*p*49 " 9 " , 7 " " " "
" 5*p*49 " 16 " , 7 " " " "
" 6*p*49 " 11 " , 5 " " " "
" 7*p*49 " 16 " , 4 " " " "

In number 14*p*49 fifty unincubated and faunated termites had been placed with the ten incubated ones and only two individuals with the right antenna cut off were taken from this vial, though others were present and might have been taken. Nearly all of the fifty unincubated individuals were alive. Now the termites with the right antenna removed which were taken from vials 3, 4, 5, 6, 7, and 14*p*49 to be counted were placed in separate vials 1–6*p*88 with a label on each vial designating the source. Whatman's filter paper was given them for food. They were observed regularly. All are alive now, 2/7/23,[1] five weeks from the time they were separated from the unincubated. Two of the individuals in 3*p*49 were examined and were heavily infected with protozoa.

After association for several weeks with termites harboring protozoa the defaunated termites regain their protozoa and, at the same time, their ability to live on a diet of pure cellulose or wood. Now, the important question is, how did the termites regain their ability to make use of pure cellulose or wood as food? More specifically, why do they now possess cellulase—and, perhaps, cellobiase? When the protozoa are removed from a termite's intestine, the ability to make use of pure cellulose or wood is lost, but when they are replaced, this ability reappears. Do the cells of the termite possess cellulase and cellobiase? It seems that they do not. Then where may these enzymes be found? The fact that there presence can only be demonstrated when the protozoa are present in the intestine of the host seems to indicate that the protozoa possibly possess them. If the protozoa do possess these enzymes, and the host does not, the way in which they aid their host is plain.

It seems evident that the protozoa aid their host in some way to live on cellulose and wood, for it has been shown (Table III.) that defaunated termites can live indefinitely on the digestion products of pure cellulose or wood (humus). It now becomes

[1] Many are alive now 4/10/23.

highly probable that the protozoa aid their host in the digestion of pure cellulose and wood, since the host is able to live indefinitely on either of these substances only when it harbors the protozoa. But how do the protozoa aid their host in the digestion of wood? Mechanically, or by furnishing enzymes, as suggested above, which the host itself does not possess.

It is possible that the protozoa mechanically aid their host since the intestine is completely gorged with them. The intestinal cells of the termite may secrete enzymes such as cellulase and cellobiase, only when stimulated by actual contact with vast numbers of intestinal protozoa. Pavlovsky and Zarin (1922) in a study of the ferments in the alimentary canal of the bee, *Apis mellifera*, found catalase in the stomach and large intestine in winter only. Three hours after the first flight in spring, catalase was present in a very small amount, and two days later, not a trace was left. These authors claim that the discharge of catalase in the rectum depends upon the accumulation of feces in it during hibernation. The bee has no need for this enzyme except during hibernation, when it seems to regulate the different oxidizing processes and destroys the surplus peroxides as they accumulate. The production of any enzyme may be explained either as a "hunger phenomenon" or due to favorable conditions of nutrition, the latter being, perhaps, the better explanation. Young (1918), in his work on inulase, showed that stimulation to increased production of this enzyme was dependent upon a direct chemical stimulus—substances present in the medium. It has been impossible to extract cellulase or cellobiase from either those termites harboring protozoa or those not harboring protozoa (defaunated termites). Pringsheim (1912) claims that cellulase is an endo-enzyme and is secreted from the cell when stimulated by direct contact with cellulose. This perhaps explains my extraction failures.

The other way the protozoa may aid their host is to digest the wood for it. Since it has been shown that defaunated termites cannot digest wood, the experiments of Buscalioni and Comes (1910), where these investigators demonstrated that the wood particles ingested by the protozoa are digested, are now of great value, though they were worthless so long as it was not known that the host could not digest wood without the protozoa. There

are many reasons for thinking that the protozoa digest the wood for their host. In *Reticulitermes flavipes* practically all the wood particles which reach the hind-intestine are immediately ingested by the protozoa. Hundreds of individuals of this species have been examined to note if wood particles were ever present in a very great quantity in the intestine, and in only a few instances were they ever found in abundance. Perhaps, directly after the termite has taken a meal, wood particles may be quite plentiful, in the intestine, but it is certainly not long before nearly all of these particles of wood are taken into the bodies of the protozoa. In *Termopsis* many wood particles are free in the intestine, though the wood ingesting protozoa harbored by this genus always have their bodies filled with wood particles to the same extent as do the wood ingesting protozoa harbored by *Reticulitermes*. Quite a bit of woody material is present in the expressed pellets of excrement of *Termopsis*, whereas *Reticulitermes*, on the contrary has a liquid excrement.

Of course the mere fact that the protozoa take the particles of wood into their bodies does not mean that they in any way alter these substances, for many protozoa ingest substances which they cannot use as food. Not all of the termite protozoa ingest solid particles of wood for food, or at all, though most of them do. Among the protozoa harbored by *Termopsis* sp., of Ashland, Oregon, *Streblomastix strix* does not ingest the wood particles, and this organism never gives a glycogen reaction. *Streblomastix strix*, then, may get its nourishment from the digestion products of the other protozoa which *Termopsis* harbors, such as *Trichonympha campanula*, which ingests great quantities of wood particles and always gives a very clear cut and definite glycogen reaction, provided its host has not been wood starved; or it may derive its nourishment directly from the products which its host has assimilated, in which case it is a true parasite. If this protozoön gets its nourishment from the digestion products of other protozoa present in the intestine of its host, just in the same way that the termite gets its nourishment, *Streblomastix strix*, then, may be a commensal from the viewpoint of the termite, but from the viewpoint of its relation to the wood digesting protozoa, it is a true parasite, since it in no way aids the wood digesting protozoa, whereas the termite does aid them since it

furnishes the wood which they digest. Of course *Streblomastix strix* may use both the products assimilated by the termite and the products present in the termite's intestine as a result of the digestion of wood by other protozoa, which products have not been assimilated by the termite. Those individuals of *Trichonympha campanula* containing the greatest quantity of wood particles in their bodies give the most distinct glycogen reaction. The reaction is much more pronounced in the posterior half of the body where most of the ingested wood remains. Another form, *Trichomonas termitis* (*Trichomonas* because this organism has an undulating membrane, axostyle and three flagella and is not the same organism as *Trichomitus termitidis* which Kofoid and Swezy (1919) described from *Termopsis angusticollis* of Berkeley, California), ingests wood particles but does not give the glycogen reaction except in a very few instances. As a matter of fact, about one individual in a hundred examined will give a slight glycogen reaction. This organism probably does not make use of the wood particles, but, perhaps, ingests them while feeding on bacteria, since it has a cytostome, an organelle which *Trichonympha*, *Dinenympha*, *Streblomastix*, *Pyrsonympha*, and most of the other genera of termite protozoa, do not possess.

Among the protozoa harbored by *Reticulitermes flavipes*, *Trichonympha agilis* and *Pyrsonympha vertens* ingest wood particles and give a glycogen reaction. Several of the species of *Dinenympha* do not ingest wood particles or give a glycogen reaction. When these termites (*Termopsis* and *Reticulitermes*) are wood starved, the protozoa (*Trichonympha campanula*, *T. agilis*, *Pyrsonympha*, etc.) that give the glycogen reaction always die off first. Sometimes practically all of these forms are dead before any diminution in the other forms occurs. *Pyrsonympha* is probably of great value to its host owing to its peculiar habit of attaching itself to the chitinous layer investing the inner surface of the intestine. Individuals of this genus, instead of passing out of the intestine with the feces when they die, remain attached to it long after death, and thus the food which they have prepared for their host is kept in the intestine until the host has an opportunity to use it. The attachment is, perhaps, also quite useful to the protozoön, since this organism is not provided with numerous flagella as is *Trichonympha* and by

means of the attachment apparatus it is thus enabled to maintain its position in the intestine in the complex struggle for existence among the many species of protozoa which completely fill the lumen of the termite's intestine. No doubt *Pyrsonympha*, *Trichonympha* and others, die by the hundreds daily, and thus give over to their host many substances which they have obtained from the wood particles. That they give sugars,[1] such as xylose, may be shown by testing with phloroglucinol and hydrochloric acid. But it is not the intention of the writer to show in this paper the relation of each species of protozoa to its host. Not all of the protozoa harbored by the two hosts, *Termopsis* and *Reticulitermes*, have been mentioned, and the protozoa of other termite genera have not been mentioned at all. In a later paper this question will be considered in more detail.

Unwilling to conclude definitely that the protozoa were entirely responsible for the digestion of cellulose, the other microörganisms harbored by *Reticulitermes flavipes* were studied. Bacteria were sometimes numerous, and these were studied in many ways. Incubation at 36° C. for 24 hours does not seem to affect their numbers. All the known methods, aërobic and anaërobic, for isolating cellulose decomposing bacteria were given more than fifty trials, but, since the results of all these experiments were negative, no tabulation has been made. An inorganic medium composed of

| | |
|---|---|
| $K_2HPO_4$ | 1.00 gram |
| $MgSO_4$ | 0.50 " |
| $KCl$ | 0.50 " |
| $FeSO_4$ | 0.01 " |
| $NaNO_3$ | 2.00 " |
| $H_2O$ | 1000.00 cc. |

to which cellulose was added in two forms: a small piece of Whatman's filter paper was placed in the test-tubes containing the inorganic medium and a 0.5 per cent. cellulose suspension was added to the inorganic medium. This was sterilized in the usual way and the inoculations made. Then the cellulose suspension and inorganic medium plus agar sufficient to make a

[1] Provided the conclusions of Buscalioni and Comes (1910) regarding the phloroglucinol reaction are accepted. For a discussion of this reaction see page 205–6.

solid medium were used. When cellulose was the only carbohydrate present, no growth ever occurred; but when other carbohydrates, such as dextrose, were present a rapid growth took place. For more information regarding the methods used in studying cellulose decomposing microörganisms the reader is referred to Waksman (1919 and 1922 [1]), Kellerman (1913), McBeth (1913 and 1916), Brown (1915 and 1917), Schmitz (1919), and others. After employing all of these methods it was concluded that no cellulose digesting bacteria are harbored by *Reticulitermes flavipes*, since more than one hundred individual attempts to isolate cellulose decomposing bacteria met with failure, even after the cultures were more than two months old. It was an easy matter to isolate such bacteria from the soil and from cow chips.

An attempt was also made to isolate cellulose decomposing moulds and actinomycetes from termites, and this attempt was a failure. This experiment was repeated ten times with the same result each time.

None of the known cellulose-digesting bacteria, moulds and actinomycetes dies at 36° C. As a matter of fact this is the optimum temperature for many of these organisms. In some instances the cellulose is decomposed much more rapidly at even a higher temperature.

These negative results indicate that *Reticulitermes flavipes* harbors no bacteria, moulds or actinomycetes capable of decomposing cellulose.

The most logical conclusion, then, from the results of all the experiments carried out, is that the protozoa actually aid their host by digesting the wood—or more specifically by breaking up the cellulose molecule and freeing many substances which are bound to it in some way—for it, because the host itself cannot digest cellulose. The protozoa can digest cellulose.

The relationship, then, between *Reticulitermes flavipes* and some of its intestinal protozoa, particularly *Trichonympha* and *Pyrsonympha*, is one of symbiosis. The termites are able to maintain themselves on their normal diet of wood only when they harbor protozoa to digest the wood for them. The protozoa are harbored only when their host eats wood or the excreta of

[1] This paper contains 957 references.

other individuals containing wood and protozoa. The larvæ must become infested with protozoa, by eating the feces of older and infested members of the colony, before they can maintain themselves on a strict wood diet. The host procures the wood for the parasite, and the parasite digests it for itself and for its host. Each is a servant to the other; the protozoa are dependent on the termites for food and lodging, and the termites are dependent on the protozoa for the protozoal cellulose digestion products.

## GENERAL DISCUSSION.

The number of protozoa present in a sigle host is truly enormous. Here we have an animal whose intestine is completely filled with flagellate protozoa, the majority of which live in a symbiotic relation to their host. Perhaps many—if not all—intestinal flagellates—human as well as animal—are harmless to their host. At least their presence does not mean anything—harmful or otherwise. Only careful and painstaking investigation will reveal their relation to their host.

The examples of symbiosis in nature hitherto described, such as algæ and fungi (forming lichens), hydra and Zoöchlorellæ, sea-anemone and hermit crab, are well known. These relationships are much closer than such relationships as ants and aphids, insects and flowers, etc. The symbiosis exhibited by termites and their intestinal protozoa is as real or true as any yet described. How such a relationship was developed is an extremely interesting but very difficult question to consider—or even theorize [1]

[1] Since none of the protozoa of termites are known to occur elsewhere it is interesting to speculate on their origin. Where did these insects get them? The ability of soil protozoa to digest cellulose has not been studied and little is known regarding the protozoa of plants. If there are protozoa parasitic on plants, or protozoa in the soil, capable of digesting cellulose, it is possible that some of them became inhabitants of termites. It may be that the symbiosis was established in this way. These insects, before harboring protozoa, fed on soil and wood and, perhaps, were able to utilize some of the substances in the wood and many of those in the soil. The intestine of termites, feeding on practically the same food that the protozoa free in nature feed on, is certainly a much more constant—and hence better—environment for the protozoa than soil, or even plants. But, if the termites once possessed the ability to digest cellulose, why did they lose it? If the acquired parasites had been digesting cellulose longer than the termites and could do it better, *i.e.*, more completely, this may be responsible for the loss of the ability to digest cellulose. The insects probably lost their ability to secrete enzymes, such as cellulase and cellobiase, because it became unnecessary for them to do it. However, it is also just as reasonable to suppose that the termites never possessed the ability to digest cellulose, since so few animals do.

on—for it has undoubtedly been established a very long time.

The ability of animals to live indefinitely with a sterile intestine is still a live, debatable, question. Osborn and Mendel (1914*b*) have shown that microörganisms are of value to higher animals as elaborators of protein from non-protein stbstances ingested. Armsby (1911) showed that non-protein substances are a source of protein to herbivorous animals, probably due to the formation of digestable bacterial protein in the digestive tract. There is certainly a very small amount of protein in wood, and it may be that termite protozoa aid their host in some such fashion as intestinal bacteria aid their host. Kianigin (1917) reopened this question by a review of all the literature. The greatest quantity of microorganisms—in the case of mammals and other vertebrates—is located in the nondigestive portions of the alimentary canal. Also the increasing number of animals which may be raised aseptically indicates that an aseptic existence may be possible in the majority of cases. Bacteria carry decomposition to a lower level, yielding unassimilable substances such as methane, carbon dioxide, indol, skatol, phenol, cresol, etc. Perhaps the chief beneficial rôle of the intestinal flora is the synthetic powers of the microörganisms.

But the relation of many insects to microörganisms is a different question, and at least from one viewpoint, if from no other, a more interesting one and also one which may be studied more satisfactorily. Baumberger (1919) has shown "that *Drosophila* living in fermenting fruit are dependent for their food supply on the synthetic and absorptive powers of yeast cells." In a similar manner his study of "the relation of *Musca domestica* to manure, of *Desmometopa* to decaying meat, and of *Sciava* and *Tyroglyphus* to decaying wood shows clearly that these arthropods also feed

Another possibility, and perhaps a more plausible one, is that the termites before they became infected with protozoa, probably fed on humus. Some wood was ingested—though not digested—along with the humus. The protozoa were taken in and found the environment suitable. Rapid multiplication and few deaths in the more suitable environment, soon filled the termites with them. The wood feeding habit of the termites gradually increased. At present every conceivable step from non-wood feeding termites to strict wood feeders, is well illustrated in the various termites.

But what happened to the ancestors of pre-termite protozoa—the organisms which lived in the soil or in wood? They have probably evolved along another line, in an opposite direction from those individuals who became inhabitants of termites.

on microörganisms." However, microörganisms are not associated with these insects as symbionts—certainly not in the same sense or degree that termite protozoa are associated with their host—but as food.

The nitrogen content of birch-wood is very high as compared with that of other woods. It is 0.108 per cent. In many woods it runs as low as 0.08 per cent. From the work of Baumberger (1919) and others, it is evident that many insects, due to the low protein content of the substratum on which they live and feed, are dependent on microörganisms for the proteins which they contain. Since such a small amount of protein is present in the food of termites it is quite likely that they, too, are dependent on microörganisms for a part of their proteins. Fungi may often be seen in the intestine of many termites.

The relation of ambrosia beetles to fungi has been studied by Hedgcock (1906) and others, and the fungus-growing habits of the Termitidæ have been studied by many investigators, chief of which are Doflein (1905 and 1906), Petch (1906) and Escherich (1909), but the exact relation of these insects to the fungi is not known. The fungi may simply furnish food (protein) for the insects, and, on the other hand, they may elaborate substances from the wood and grasses which the beetles and termites cannot elaborate, which substances are very important in the insects' metabolism.

Internal symbionts (yeasts) have been described from the beetle *Anobium paniceum*, by Karawaiew (1899) and Escherich (1900), and Portier (1905) claims that a micrococcus and a fungus live symbiotically with the caterpillar *Neptiluca*, but in no instance have intestinal flagellates been shown to live symbiotically with their host. The flagellates living in termites are the first example of such a relationship.

As a rule the second and third reproductive forms do not harbor intestinal protozoa. Why? For one of two reasons perhaps: (1) the liquid diet upon which these individuals are fed kills the protozoa; (2) the failure of the host to eat wood causes the death of the protozoa. There is more evidence in favor of the latter view. In the first form reproductive adults protozoa are usually present—though never in abundance as in workers and soldiers—and this form eats some, though never as much

wood as the workers and soldiers. When workers and soldiers are wood starved the wood ingesting protozoa which they harbor die first. When workers are fed cow chips the protozoa disappear in three to four weeks. But here, as in the case of saliva, it is impossible to determine whether the change in the intestinal content brought about by the change of food, results in the loss of protozoa, or whether the failure of the host to provide the protozoa with wood results in their death. Since there is a great deal of cellulose in the cow chips it is more likely that the change in the intestinal content is responsible for the death of the protozoa. The bacteria of the cow chips do not kill the protozoa, for sterile cow chips are just as effective in removing them. This question is now being attacked from many angles and it is hoped that a more definite answer may result.

There is considerable difference of opinion regarding the extent to which cellulose is utilized in the animal organism. Very little is really known about the mechanism which vertebrates—and most, if not all, invertebrates too—employ in making use of cellulose. It is interesting in this connection to compare the views expressed by two prominent physiological chemists. Hawk (1921) states that many of the herbivora use as much as 25 per cent. of the total ingested cellulose, that less than 5 per cent. is used by dogs and the amount used by man is "too small for it to play a rôle of importance in the diet of a normal individual." He says: "In neither man nor the lower animals has there been demonstrated any formation of sugar or glycogen from cellulose." Von Furth (1916) states that 30–70 per cent. of the cellulose eaten by herbivorous domestic animals is digested, and that man digests about 50 per cent. of the cellulose which he consumes, and in cases of habitual constipation he may digest as much as 80 per cent. The filtered mammalian intestinal content is inactive to cellulose (Pringsheim, 1919). Pringsheim (1919) also claims that no cellobiose-splitting enzymes are present in any of the organs of cattle. Biery (1914), Biery and Giaja (1912) and Billard (1914) report the presence of cellulose-splitting enzymes in hepatic secretions of certain mollusks and crustaceans. Harrington (1921) claims to have demonstrated the presence of cellulose-splitting enzymes in *Teredo*. Dore and Miller (1923) made a comparative analysis of the wood eaten and the borings

passed by *Teredo navalis*, and, as a result of the analyses, these investigators conclude that "the wood lost about 80 per cent. of its cellulose, and from 15–56 per cent of its hemicelluloses, including from 11 to 40 per cent furfural yielding constituents such as pentosans, etc.," during its passage through the animals' digestive tracts. They state further that "The simplest explanation of the disappearance of this carbohydrate material is that the cellulose and hemicelluloses of wood are partly digested by the teredo and probably hydrolyzed to simple carbohydrates which the animals can use." In all these investigations it should be noted that many substances other than cellulose were present. In no instance was cellulose alone fed the animals. It would be very interesting, indeed, to study the microörganisms of these crustaceans and mollusks. No mention is made by these investigators regarding the possibility of microörganismal cellulose digestion.

Recently a cellulose-digesting anaërobic bacterium has been isolated from 60 per cent of the human stools examined (Khouvine-Delaunay, 1922), and it is quite possible that future researches will reveal that intestinal microörganisms perhaps play a more important part in cellulose digestion than has previously been thought. Intestinal bacteria and fungi quite often aid their vertebrate and invertebrate hosts in the digestion of cellulose and, since it has been shown in the present investigation that intestinal protozoa can digest cellulose, it is now possible that the Infusoria, such as *Diplodinium*, *Entodinium*, *Butschlia*, *Isotricha*, *Dasytricha* and *Ophryoscolex*, harbored by ruminants, notably the ox, goat, sheep, camel and reindeer, may aid their hosts in the digestion of cellulose and hemicellulose. The Equidæ also harbor similar Infusoria.

## General Summary.

1. There are four families of termites and all the species and genera of three of them, Kalotermitidæ, Rhinotermitidæ and Mastotermitidæ, that have been examined have been found to harbor enormous numbers of intestinal protozoa. No termite of the other family, Termitidæ, has been found to harbor intestinal protozoa.

2. The principal food of protozoa harboring termites is wood and the principal compound in the wood which the termites use

is cellulose. This was demonstrated by keeping termites alive and active indefinitely on a cellulose diet.

3. The protozoa harbored are *all* killed off by incubation at 36° C. for 24 hours, while the termites apparently are not injured at all by the incubation.

4. The incubated and defaunated (with the protozoan fauna removed) termites die within 10-20 days, on the average, after incubation, if fed their normal diet of wood.

5. When the incubated and defaunated termites are fed digested wood (*e.g.*,humus) or fungus digested cellulose, they live indefinitely.

6. The death of the incubated and defaunated termites is not due to the incubation *per se*, but to an inability to digest wood.

7. When the incubated and defaunated termites are reinfected with protozoa their ability to utilize wood, their normal diet, reappears, and they live indefinitely.

8. The removal, then, of the protozoa seems to be responsible for the loss of the ability to utilize wood as food. To determine this question the ability of the bacteria, fungi and protozoa, harbored by termites, to digest wood or pure cellulose was carefully studied. It was found that the bacteria and fungi could not digest cellulose, but that some of the protozoa could.

9. Now, since the termites die in 10–20 days, if fed a wood diet, after the protozoa have been removed from them because they cannot digest their food (wood), as shown by the fact that they do not die, but live indefinitely,when fed digested wood or when reinfected with protozoa and fed wood, and since the protozoa do digest the wood particles which they take into their bodies, it is highly probable, if not certain, that the termites are dependent on the protozoa to digest their food for them.

10. Not all of the protozoa harbored by *Reticulitermes flavipes* digest wood particles. Some of them either live at the expense of the wood digesting protozoa or their host, or both.

11. The protozoa receive from the termites food and lodging, for which they give in return protozoal wood digestion products.

12. The relationship between some of the protozoa, particularly *Trichonympha* and *Pyrsonympha*, and their host, *Reticulitermes flavipes*, is one of symbiosis.

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# THE CELLULAR ELEMENTS IN THE PERIVISCERAL FLUID OF ECHINODERMS.

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A consideration of the cellular elements in an animal group, particularly those cellular elements which are present in cavities derived from the primitive cœlom, should include references to the origin of these cells and any other factors which may be concerned with their modifications, such as the habits, the powers of regeneration and the topographical anatomy of the organ systems of the animals examined. It is generally conceded from the evidences of palæontology and comparative embryology that the Echinoderms of to-day are a fairly ancient group which have been able to adapt themselves to diverse environmental changes. The same pentaradial symmetry is present in all of them, although masked in some, but the principal variations which are characteristic of the classes are concerned primarily with the character of the body wall and secondarily with the distribution of the breathing organs. In the Echinoderms we can distinguish three types of organization of the body wall and breathing organs: the first type is characterized by a fairly flexible body and diffuse breathing organs (*e.g.*, Asteroidea and Ophiuroïdea), the second by a rigid test and limited breathing organs (*e.g.*, Echinoidea), and the third by a well-developed muscular body wall and limited breathing organs (*e.g.*, Holothuroidea). In all of the classes except the Holothuroidea, the movements of the body are very sluggish, consequently the oxygen requirements for muscular activity are very low and a system for rapid transfer of oxygen is not needed. But in the majority of the Holothuroidea, the development of muscle necessitates a great available supply of free oxygen and a mechanism for carrying this oxygen must be present. Therefore one phase of this investigation will attempt to correlate the appearance of different types of cells in the perivisceral fluid with the character of the body wall, the distribution of the breathing organs and the movements of the body as a whole.

We know that the Echinoderms have great powers of regeneration and may conclude, therefore, that all of the cells in the body are very labile, but the question arises as to which cells in the perivisceral fluid are the most generalized. Cells which could be regarded as the most generalized would be those which are constant in the perivisceral fluid of all of the Echinoderms and which are observed to have diversified functions; those cells which under necessity of local needs remove foreign material, wornout fragments of cellular origin and which could give rise to modified cells. Another phase of this investigation, then, is to determine if there are any such cells in the perivisceral fluid, and if so, what is the nature of their activities.

## I. Material and Methods.

The material used in this investigation was collected in the vicinity of the Puget Sound Biological Station, Friday Harbor, Washington. Representatives of the four classes of Echinoderms found in this area were used. The following are the species which have been examined:

Class I. Asteroidea.

1. *Evasterias troschelii.*
2. *Solaster simpsonii.*
3. *Dermaster imbricata.*
4. *Pisaster ochraceus.*
5. *Leptasterias hexactis.*
6. *Henricia leviuscula.*
7. *Pycnopodia helianthoides.*

Class II. Ophiuroidea.

1. *Ophiopholis aculeata.*

Class III. Echinoidea.

1. *Strongylocentrotus drobachiensis.*
2. *Strongylocentrotus franciscanus.*
3. *Echinarachnius eccentricus.*

Class IV. Holothuroidea.

1. *Cucumaria japonica.*
2. *Cucumaria chronjhelmii.*
3. *Stichopus californicus.*

Perivisceral fluid from individuals of each of the above species was drawn from the perivisceral cavity and studied by the hanging drop method. In studying the phagocytic activity of the cells, a concentrated suspension of finely granulate carmine or india ink in seawater was used. This suspension was injected into the perivisceral cavity through a minute opening in the body wall by means of a delicate hypodermic needle. The amount of suspension injected varied with the size of the animal, but it was found that the usual dose sufficient to affect the phagocytes was 8 cc. The injected animals were kept in a live box for a day, so that there would be time for thorough ingestion of the particles.

The clotting activities of the cellular elements were studied in drops of perivisceral fluid which had been allowed to stand for varied lengths of time.

A saturated solution of seawater and methylene blue, another of seawater and neutral red were made and allowed to stand for several days before using. The supernatant solution which was free from particulate matter was decanted off and the solution used drop for drop with the perivisceral fluid. These stains were used for intravitam staining in certain phases of the investigation since they were found to be specific for the vacuoles of the leucocytes.

## II. Observations.

### 1. *The Leucocytes.*

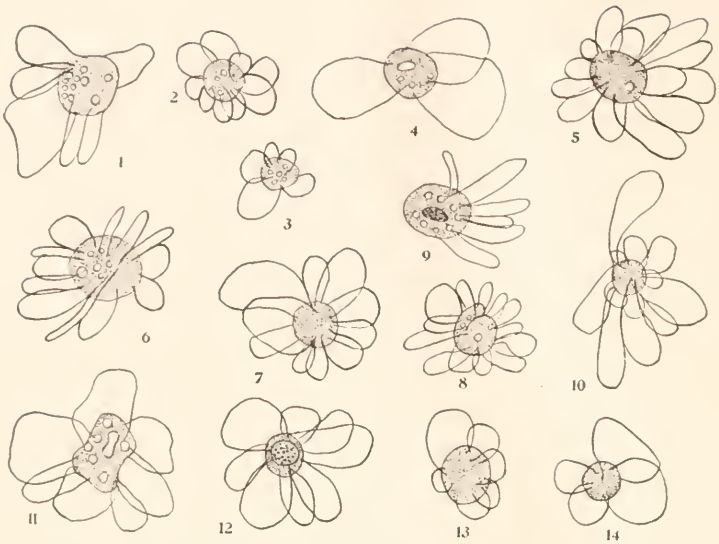
In his paper on the leucocytes of the invertebrates, Goodrich ('19) called attention to the fact that the leucocytes of *Asteracanthion glacialis* are characterized by the presence of extensive membranous processes of the ectoplasm. He says, "The freely projecting pseudopodia usually described are either figured from optical sections of the folded membranes or from cells which have produced them under abnormal conditions. These pseudopodia may be present on cells in the fluid withdrawn from the body and which has been allowed to stand, and are probably derived from preëxisting membranes." Goodrich calls all of the cells leucocytes, making no distinctions.

In *Arbacia* (Kindred, '21) I observed the formation of a syncytium in the perivisceral fluid by the anastomosis of filiform processes which had been derived from the membranous flaps of the

leucocytes, thus supporting Goodrich's assumption. Théel ('21), however, in a review of his descriptions of the types of amœbocytes in the perivisceral fluid of *Asterias rubens* and *Parechinus miliaris*, states that he distinguished two types of amœbocytes in the cœlomic cavity of these forms, the "white or hyaline plasma-amœbocytes" and the "bladder amœbocytes." These descriptions were not referred to by Goodrich nor myself although they appeared in Swedish prior to both of our papers. According to Théel the two types of amœbocytes are very dissimilar, the "bladder amœbocytes" not being flattened nor spread out on the surface of the glass when a drop of the fluid is placed on a glass slide, but remaining thick and compact with the bladder lying in several superimposed layers. On the other hand, the "hyaline plasma-amœbocytes" in the fresh fluid are characterized by the possession of a concentrated cell body with longer or shorter pseudopodia. Théel expresses doubt as to whether or not one type may be derived from the other. Following the terminology used by Goodrich, I am calling the cells with the membranous flaps leucocytes, and regarding Théel's "hyaline plasma-amœbocytes" as a secondary phase of the leucocytes from my observations on the formation of syncytia. Théel ('21) in expressing an opinion as to the possibility of morphological changes in such cells says: "However, it may be presumed that the character of the surrounding medium may play an important part in that, and above all movement and relative stillness, the former preventing and the latter forwarding the process of transmutation. If for instance an amœbocyte leaves the cœlomic cavity in order to immigrate into the tissues of the body wall, it must necessarily undergo certain changes of form. When a cell passes over from a passive drift to an active motion, its primitive globular configuration must be exchanged for another and accommodated to creeping about."

Therefore, the presence of two forms of cells in the freshly drawn drop does not of necessity mean that because of this occurrence we are dealing with two distinct types instead of an active and passive form of one type of cell. It is reasonable to suppose that these cells have a cycle of life and that as they become older this change to a more passive condition leads to a change in form. Since the activities of these cells *are* comparable

to the activities of the leucocytes in other animals, they are so termed.




FIGS. 1–14. Active leucocytes of the Echinoderms, camera lucida, × 650. 1. *Leptasterias hexactis*. 2. *Solaster simpsonii*. 3. *Dermaster imbricata*. 4. *Pycnopodia helianthoides*. 5. *Pisaster ochraceus*. 6. *Henricia leviuscula*. 7. *Evasterias troschelii*. 8. *Strongylocentrotus drobachiensis*. 9. *Ophiopholis aculeata*. 10. *S. franciscanus*. 11. *Echinarachnius eccentricus*. 12. *Cucumaria japonica*. 13. *C. chronjhelmii*. 14. *Stichopus californicus*.

Text-figures 1–14 are camera lucida drawings of the active phases of the leucocytes (" bladder amœbocytes" of Théel) of the whole series of Echinoderms studied. Examination of these figures shows the general morphological similarity of these cells to each other, the only distinct difference being that of size, which varies from 7–14 microns in endoplasmic diameter. In all of the cells it will be noted that the ectoplasm is clearly marked off from the endoplasm and is produced into a varying number of rapidly changing delicate flaps. These flaps are constantly being withdrawn and extended and may be regarded as modified pseudopodia. By means of these flaps the leucocyte progresses slowly through the fluid. That the surface of the cell is covered with a sticky fluid is evidenced by the manner in which particulate matter adheres to the flaps. When the flaps are withdrawn the particles which adhere to them are ingested.

The endoplasm of nearly all of the active leucocytes is granular and opaque, the exceptions to this opacity are found in the leucocytes of *Ophiopholis aculeata* (Fig. 9) and *Cucumaria japonica* (Fig. 12). In these leucocytes the nucleus with its content of large chromatin granules is easily discernible. The endoplasm usually contains a varying number of hyaline vacuoles which stain blue with the methylene blue-seawater solution and red with the neutral red-seawater solution.

The active leucocyte is a phagocyte and is always found loaded with carmine or india ink particles when these substances are introduced into the perivisceral cavity. In the Holothuroidea these phagocytes were observed to deposit the india ink particles in the skin (Schultz, '95). Awerinzew ('11) considers that the color of the test in some of the Echinoidea is due to this phagocytic activity of "amœbocytes" and that the color difference in varieties of *S. drobachiensis* is dependent upon the color of the food, the pigments of which are taken up by the "amœbocytes" and carried to the skin. Since the leucocytes are the only types of cells in the perivisceral fluid which are phagocytic, they are without doubt the phagocytic "amœbocytes" whose activities are described by these and other authors. A further function of the phagocytes is the removal of germ cells remaining in the gonads after the bulk of the gametes have been shed (Caullery and Siedlicki, '03). Cernovodeanu and Henri ('06) observed that bacteria injected into the body cavity of sea urchins were taken up by "amœbocytes" with long pseudopodia, cells which are doubtless leucocytes. Since mention has been made above of the relation of the leucocytes to the transfer of food products, it is pertinent to enquire as to further observations of their participation in this phase of vital activity.

Cuenot ('91b), one of the first to call attention to this relation, assumed that the substances passed from the intestinal cells into the intestinal lacunæ are taken up by the "amœbocytes" and stored up in them to be carried to other parts of the body. The "amœbocytes" which are so concerned become metamorphosed into "amœbocytes with spherules." Frenzel ('92) was of the opinion that the "amœbocytes" pushed between the epithelial cells of the intestine and into its lumen where they disintegrated and their remnants acted as a digestive ferment. Enriques ('02)

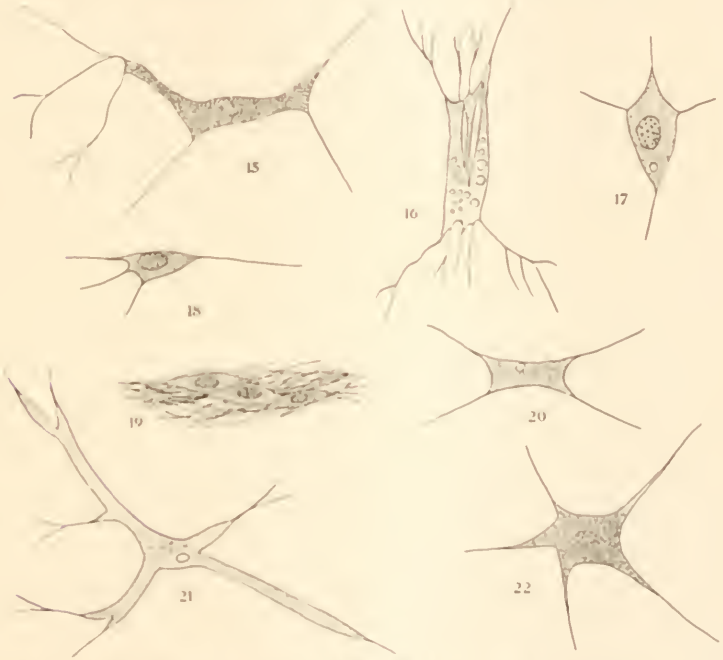
thought that digestive substances were carried from the rete mirabile peritoneum in the Holothuroidea to the stomach epithelium by "amœbocytes." Thus there are several conceptions concerning the relations of the leucocytes to the digestive activities which need further investigation.

As evidenced by their tendency to remove foreign particles from the body cavity the leucocytes may be regarded as excretory agents and further observations as to their excretory activities should be considered. Durham ('88) and Chapeaux ('93) observed that the phagocytic cells leave the body through the papulæ in the Asteroidea. I have observed such a migration in *Leptasterias hexactis*, in which, after injection with carmine, the papulæ are reddish and a smear from the outer surface reveals a number of leucocytes laden with carmine particles. "Amœbocytes" (particular type not stated) have been observed to leave the body cavity of the Holothuroidea by diapedesis through the walls of the branchial tree into its lumen and thence to the outside (Herouard, '95; Schultz, '95). Therefore, there is evidence that the exit of the phagocytes in the Echinoderms is through the body wall.

The exact relation of the leucocytes in the removal of waste substances from the tissues has not been proven, but Delage and Herouard ('03) thought that substances absorbed from the tissues by "amœbocytes" are reprecipitated in them in the form of granules and may possibly give rise to the various "amœbocytes with spherules." List ('97) pointed out earlier that substances absorbed by the "amœbocytes" may be the cause of the development of a crystalloid in the nucleus of cells of this type, which by growth causes a degeneration and finally the destruction of the cell. Thus the accumulations of crystalloids observed scattered throughout the body of various Echinoderms may be regarded as the remnants of degenerate excretory "amœbocytes."

Another activity of the active leucocytes is the formation of plasmodial masses which are very numerous in any drop of perivisceral fluid. That these plasmodia are formed by the fusion of active leycocytes has been observed frequently and plasmodial formation is one of the activities which distinguishes the active from the passive phase of the leucocyte and from other cells in the perivisceral fluid.

In addition to the active leucocytes there are always present, even in a drop of freshly drawn perivisceral fluid, numbers of flattened cells with filiform processes which float passively through the fluid (Figs. 15–22). The endoplasm of these cells is granulated, opaque and vacuolated, as is the endoplasm of the active leucocytes and it reacts the same to methylene blue-seawater or neutral red-seawater solutions, *i.e.*, the vacuoles stain blue and red respectively. Since I have observed active leuco-




FIGS. 15–22. Passive leucocytes of the Echinoderms, camera lucida, × 650. 15. *Dermaster imbricata*. 16. *Henricia leviuscula*. 17. *Cucumaria japonica*. 18. *Stichopus californicus*. 19. *Strongylocentrotus drobachiensis*. 20. *Solaster simpsonii*. 21. *Pisaster ochraceus*. 22. *Ophiopholis aculeata*.

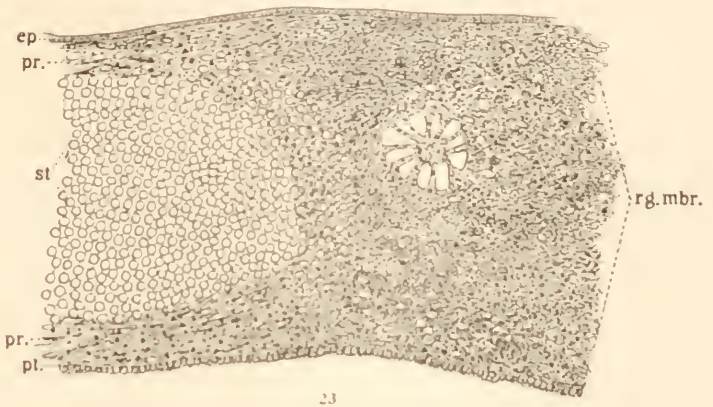
cytes changing into these in *Arbacia*, I think that they are passive phases of the leucocytes. Théel regards them as distinct in themselves and calls them "hyaline plasma-amœbocytes." Their presence in the freshly drawn fluid would indicate that they are normally present and that their occurrence is not due to abnormal conditions as Goodrich suggested. Although these cells may occur singly, they are most frequently met with as

elements of a syncytium and it is this syncytial formation which is their most valuable vital activity, in that it is concerned with the repair of injury and the replacement of lost parts in the organism. This is due to the fact that these syncytia close wounds and form the basis for the growth of the other tissues and further that these cells themselves may take part in the formation of other tissues.

Théel ('21) states that there is a fibrin-like substance which occurs in the coagulation of the cœlomic fluid in the Holothuroidea in addition to the meshwork of the fibers formed by the arborizing leucocytes. That this fibrin-like substance is extracellular is evidenced by the fact that a fibrous meshwork occurs when there are far too few cells to form such a meshwork so rapidly, by leucocytic syncytia alone. I observed this superfluity of fibers in the drops of perivisceral fluid of all of the preparations of fresh material and was at a loss to account for it. The origin of this fibrous substance is not known, although it has been suggested by Schäfer ('83) that it might have been secreted by the leucocytes. That it does not coagulate in the fluid in the perivisceral cavity is due to the various ciliated cells which keep the fluid in motion (Cuenot, '01). This movement of the perivisceral fluid does not prevent the formation of plasmodia or syncytia, but it does seem to inhibit the fibrin-like coagulum.

From the observations of Théel and others it is well known that in the development of the test of the Echinoidea the leucocytes form syncytia within which is secreted (intracellularly) the spicules which fuse and form the stereom, a definite beam and rafter skeletal structure. Leucocytes containing spicules have been observed in the perivisceral fluid of various Echinoderms. I have observed them in the perivisceral fluid of *Henricia leviuscula* (Fig. 16). Théel from his observations on the occurrence of these cells containing spicules is of the opinion that we may presume that the skeleton of the Echinoderms is due to the activity of migratory "plasma-amœbocytes" and their syncytial fusion. This assumption may be true, but there has been no evidence presented which shows that these cells (my leucocytes) are concerned in the replacement of resected areas of the stereom. Hence in order to determine the scleroblastic activity of the leucocytes a series of resections of the test of *S. drobachiensis* were

made and the subsequent regeneration observed. But before discussing the observations the relationships of the stereom and the adjacent region should be described. Thus in a section

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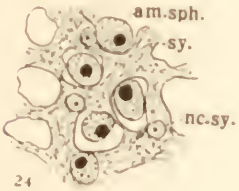


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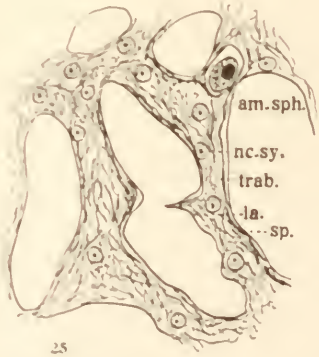


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FIG. 23. Regenerating body wall, *Strongylocentrotus drobachiensis*, vertical section, semi-diagrammatic, camera lucida, × 75.

FIG. 24. Detail of reticulum of regenerated membrane of body wall shown in Fig. 23. Semi-diagrammatic, camera lucida, × 650.

Fig. 25. Detail of prestereomal area shown in Fig. 23. Semi-diagrammatic, camera lucida, × 650. *am.sph.*, "amœbocyte with spherules"; *ep.*, epidermis; *la.*, lacuna; *nc.sy.*, nucleus of syncytium; *pr.*, prestereomal area; *pt.*, peritoneum; *rg.mb.*, regenerated membrane; *sp.*, spicule; *st.*, stereom; *sy.*, syncytium; *trab.*, trabecula.

through the body wall of *S. drobachiensis* which had been decalcified and stained the following relations of the stereom and the surrounding tissues may be observed. The epidermis (Fig. 23) appears on the outer surface of the section and beneath this there

is a thin layer of syncytial reticular connective tissue (*pr.*) which is directly continuous on its inner surface with the definitive stereom (*st.*). This syncytium is loosely organized and has large intersyncytial spaces within which are found wandering "amœbocytes with spherules." The cytoplasm of the syncytium is fibrous in appearance and the remnants of spicules could be observed in it. The traces of spicules were more marked in the region bordering on the stereom where the syncytium had the trabecular organization characteristic of the stereom. This region I have designated as the prestereomal area. The stereom is made up of I-shaped trabeculæ which are apparently joined to each other end to end, so that the whole stereom is a framework of beams and rafters with regular lacunar spaces. The substance of the stereom when observed in the unstained condition is clear crystalline in character and when stained is intensely basophilic in reaction. A prestereomal area is also found between the stereom and the peritoneum. From the organization and relation of the prestereomal areas to the stereom it is evident that the growth of the stereom takes place peripherally by the gradual deposition of skeletal material within the prestereomal trabeculæ. Nuclei with prominent nucleoli were observed in the prestereomal trabeculæ, but none were observed in the trabeculæ of the stereom. This condition would indicate that trophic activity of the cells is lost in giving rise to the stereom and that the whole cytoplasm of the syncytium becomes converted over into skeletal material while the nucleus degenerates. Now if a cut were made directly through the body wall and a piece of it removed, the cut surface would present three regions, a middle stereom region and two peripheral prestereomal areas. It is obvious that the prestereomal areas would be capable of replacing certain parts of the test, but the question arises as to whether or not these cells are aided in this regeneration by the leucocytes.

Although the body wall of several specimens of *S. drobachiensis* were resected in an attempt to answer this question, the results are far from convincing and the description of the regeneration of the test which follows is to be studied more in detail at a later date.

Eight specimens of *S. drobachiensis* were injected with carmine in seawater through a minute perforation in the peristomial mem-

brane and were allowed to remain in a live box for twenty-four hours, so that the leucocytes would have a chance to take up the carmine particles. At the end of this time, a piece of the body wall, 1 cm. square, was removed from the aboral surface of each specimen. The resected specimens were then put into a live box and observations were made over a period of four weeks upon the changes which were taking place in the resected area. When first observed it was noted that a membrane was gradually closing the opening in the body wall. This membrane was reddish in color as contrasted with the greenish color of the surrounding body wall, and it grew mesially from the margin of the opening so that the latter diminished slowly in diameter. In seven of the eight specimens the opening in the body wall was closed in two weeks. At first the membrane closing the opening was very delicate, but it gradually became firmer and in several specimens toughened. This phase of the toughening of the membrane occurred during the third week. It was then noticed that skeletal material began encroaching on the margin of the membrane which was contiguous with the original cut surface. During the fourth week the deposition of skeletal material went further and several individuals showed a portion of regenerated test. At this time the tissue closing the opening was removed from several specimens, spread out on a slide and studied in the fresh condition. The whole mass had a crimson color and was of a tangled fibrous consistency in which were apparent leucocytes containing carmine granules which had formed more or less of a syncytium, in which the cell boundaries were indistinct. Several pieces of the membrane and the adjacent body wall were preserved for sectioning in order to determine the relation of the cells to the replacement of the skeleton. These pieces were decalcified and a series of sections made.

Figure 23 is a semi-diagrammatic drawing of a vertical section through the regenerated membrane and a portion of the adjoining body wall, the structure of the latter having been discussed above. In the membrane there are several regions of differentiation. The region most distal to the test and forming the center of the membrane is thick and reddish in color, this color being due to the presence of minute particles of carmine in the cells which make up the syncytial reticulum of the membrane

(Fig. 24). The color diminishes laterally and also in the region proximal to the stereom, where the syncytium is trabeculated in the same manner as it is in the prestereomal peripheral areas (Fig. 25). Since the leucocytes were the only cells in the perivisceral fluid which were observed to be phagocytic and form syncytia it is probable that they aid the prestereomal cells in the formation of the membrane and gradually develop skeletal material for the formation of the stereom. The cytoplasm of the reticulum in the membrane in addition to the carmine particles contains fibers which are probably the remnants of decalcified spicules. The nuclei of the reticulum are round, have a distinct nucleolus and are the same type as are present in free leucocytes observed in the lacunæ of the stereom and in the prestereomal trabeculæ, so that it is probable that the leucocytes and the connective tissue cells of the prestereomal area are of the same series, except that one has become specialized for the production of the stereom under ordinary conditions of growth, whereas the leucocytes only take over this function when the body wall is injured. The only evidence for a line of demarcation between the two is in the presence of the carmine particles in those cells which make up the reticulum of the membrane. It is therefore evident that the membrane is formed by both the multiplication of the prestereomal connective tissue cells aided by the anastomosis and syncytial formation of the leucocytes which make up the bulk of the membrane. Within the spaces of the reticulum of the membrane are found large numbers of "amœbocytes with spherules" which probably carry nutrition to the cells of the syncytium, enabling them to carry out their scleroblastic function. These "amœbocytes with spherules" are very few in the region of trabecular formation adjacent to the stereom and are entirely absent from the lacunæ of the stereom, thus they seem to be massed in that region where repair is going on rapidly and the cells of which are being differentiated.

Thus in brief there are three regions present in the regenerating area. First, the syncytial region which forms the bulk of the membrane which has closed the opening in the body wall and is composed of a syncytium of leucocytes with small lacunæ, containing large numbers of "amœbocytes with spherules." Secondly a prestereomal area, definitely trabeculated, with large

lacunæ and continuous with the peripheral prestereomal areas which enclose the stereom of the adjacent region of the body wall. The "amœbocytes with spherules" are very few in this region. Thirdly, the definitive regenerated stereom which is crystalline in character, devoid of nuclei and in the lacunæ of which there are no "amœbocytes with spherules."

It is obvious that the cellular elements which form the membrane have either been derived directly from cells in the region of the prestereomal areas or from cells of the perivisceral fluid which have migrated to this area and formed syncytia. Since the leucocytes are the only cells of the perivisceral fluid which have been observed to form syncytia and since they are the cœnocytes of the original wandering mesenchymal cells, there is reason to suppose that they have some part in this process. That stereom formation does not necessarily start from the cut surface of the resected area is evidenced by the appearance of independent areas of stereom formation in the membrane (Fig. 23). A more detailed study of the relationships of the leucocytes to the regeneration of the test is to be more closely followed in a more extended series of experiments.

These observations agree in general with Théel's conclusions that the scleroblastic cells "ought to be enrolled among the true plasma-amœbocytes, though they are dissimilar as to their functional manifestations, and consequently, too, as to their chemical constitution. It is not unlikely that their quality of taking up a superfluity of inorganic salts from the surrounding medium has influenced the cells, their power of amœboid movement having been suppressed or limited." He concludes that the directions of the pseudopodia predetermine the characteristic protrusions of the crystals in *Psolus phantapus*. The crystal when formed occupies the whole of the cell except for a small space occupied by the nucleus. In the formation of the stereom in *S. drobachiensis* a similar predetermination of the organization of the skeletal elements is apparent in the prestereomal areas.

From the above observations it may be concluded that the leucocytes in both the active and passive phases are those cells which are of constant occurrence in the Echinoderms. They are phagocytic, thrombocytic and possibly scleroblastic. These cells are omnipresent in all regions of the perivisceral cavity.

From their activities they must be recognized as the most generalized cells in the perivisceral fluid since no others have been observed whose functions are so diversified. The question of their origin is a vexing one, although it was once pointed out that the dorsal organ may, in the Asteroidea, be an organ of leucoblastic function, a function which Cuenot ('01) denies this organ, claiming that it is more likely an organ for the elimination of wornout cells of the perivisceral fluid and that the "amœbocytes" (general term) are probably peritoneal in origin and may also arise from each other. In all probability the number of leucocytes increases rapidly when an animal is injured, particularly in the region of the injury, where they form the framework for the regeneration of other tissues. They may be regarded as one of the important agents in the replacement of lost parts in the Echinoderms.

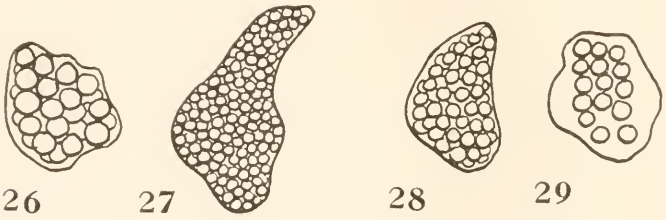



FIG. 26. "Amœbocyte with red spherules," *Strongylocentrotus drobachiensis*, cam. luc., × 1300.

FIG. 27. "Amœbocyte with red spherules," *S. franciscanus*, cam. luc., × 1300.

FIG. 28. "Amœbocyte with red spherules," *Echinarachnius eccentricus*, cam. luc., × 1300.

FIG. 29. "Amœbocyte with yellow spherules," *E. eccentricus*, cam. luc., × 1300.

### 2. *The "Amœbocytes with Spherules."*

There are two groups of "amœbocytes with spherules" present in the perivisceral fluid of the Echinoderms. One group of these is characterized by the presence of pigmented spherules in the cytoplasm and the other by colorless spherules. In both types the spherules fill the cytoplasm to such an extent that the nucleus appears merely as a light space in the center of the cell. All of the "amœbocytes with spherules" are further characterized by the presence of very blunt pseudopodia and when fluid containing them is allowed to stand they tend to assume a spherical shape.

Amœbocytes containing red spherules were observed in the

Echinoidea alone, those containing yellow spherules in the Echinoidea and some of the Holothuroidea, and those containing colorless spherules in the Ophiuroidea, Echinoidea and Holothuroidea.

In the Echinoidea, the "amœbocytes with red spherules" were most numerous and largest (12 microns in diameter) in *S. franciscanus* (Fig. 27); in *S. drobachiensis* (Fig. 26) they were less numerous and smaller (9 microns in diameter). In *Echinarachnius eccentricus* (Fig. 28) they were also less numerous and smaller (10 microns in diameter). The predominant type of pigmented amœbocyte in *E. eccentricus* was the type with yellow spherules (Fig. 29) and these were particularly massed on the peritoneum of the intestine. Scattered yellow pigmented amœbocytes were observed in *Stichopus californicus*, but no association with any organ system was noted.

The red pigment in the pigmented amœbocytes of the Echinoidea was designated echinochrome by McMunn ('85). The view of this author and Griffiths ('87) that the amœbocytes containing echinochrome are associated with oxygen transportation has never been fully accepted. Cuenot ('91b) was the first to oppose this assumption and stated that there was no change in the depth of the color when the cells were allowed to stand in the air, and that the contained pigment instead of being an oxygen-carrying pigment was stored-up food material which the cells had taken from the intestine. Further, Winterstein showed that a solution of echinochrome does not take up more oxygen than the same amount of seawater. This assertion of Winterstein's is significant, because it opens up the question as to the oxygen requirements of the Echinoderms. Of course it is obvious that all of the free oxygen used by the Echinoderms is taken from the seawater and further it has been shown that except for a difference in albuminoid content the perivisceral fluid of the Echinoderms is the same density as the seawater (Cuenot, '91). Therefore, we can assume that there is a diffusion of the seawater through the breathing organs of the Echinoderms and that the oxygen content is the same in the perivisceral fluid as it is in the outside seawater. With the exception of certain of the Holothuroidea, all of the Echinoderms have a very slight development of rapidly contractile muscle elements and hence no need for a large amount of oxygen for muscular activity, from which it

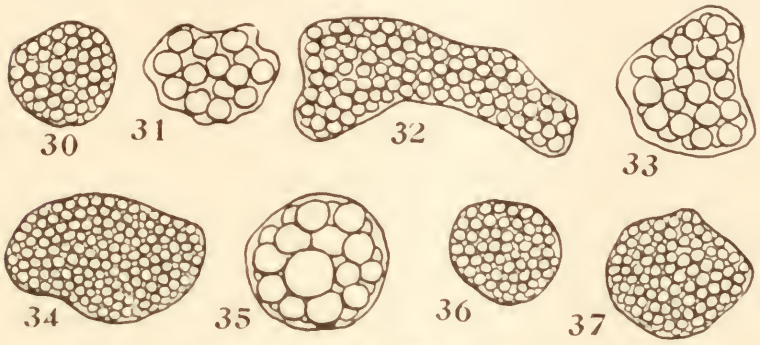
follows that in the Asteroidea, Ophiuroidea, Echinoidea and Holothuroidea with tests (*e.g.*, *Psolus*), which live in regions of high free oxygen content, due to tidal currents, there is enough oxygen in suspension in the perivisceral fluid for ordinary metabolic activity. Therefore in these forms no oxygen-carrying cells are developed and the cellular modifications which occur in the cells of the perivisceral fluid may be regarded as independent of the relations of the breathing organs. Further we would expect to find efficient oxygen-carrying cells in the Holothuroidea which have a relatively high development of muscle, for the needs of which the free oxygen content of the perivisceral fluid is not sufficient. The hemocytes are the cells which fulfill this requirement in the Holothuroidea and will be discussed later.

If the "amœbocytes with red spherules" are not to be regarded as oxygen-carrying cells, are they then related to the transfer and storage of food as suggested by Cuenot? As a partial answer to this query are the results and conclusions of Awerinzew ('11) who carried on investigations on the habitat and food relations of two varieties of *S. drobachiensis*. Awerinzew observed that the two colored varieties of this species lived in different types of environment, the green-yellow forms living on a mud and stone bottom and the red forms amongst red algæ. He assumed that the pigment in the food of the red forms was carried from the intestine to the skin and deposited there. The pigment in the perivisceral cells would then be due to the food eaten. He checked his results by injecting carmine particles in solution into the alimentary tract and found that these particles were carried to the skin, but the distinctions between the types of cells engaged in this activity were not made clear, so that it is possible that the cells were leucocytes carrying on their normal activity as phagocytes and there is no case for the pigmented cells as food carriers.

The "amœbocytes with red spherules" are far more numerous and larger in the perivisceral fluid of *S. franciscanus* than they are in *S. drobachiensis*. This fact leads to the suggestion that the color of the body wall is due to a difference in numbers of the red cells in the two species.

Since it has not been proven that the "amœbocytes with red spherules" are developed from other cells by the ingestion of

pigment from food, it may be that they are the descendants of the pigmented cells (chromatophores) of the larval Echinoidea. These cells are present in the segmentation cavity of the larval Echinoids, but I have not found any reference as to their occurrence in the larvæ of other classes. If this is true, then we are dealing with an amœbocyte which is specific in the Echinoidea and may yet be found to be derived from the colored substances characteristic of the Echinoid ovum.




FIGS. 30–37. "Amœbocytes with colorless spherules," cam. luc., × 1300. 30. *Ophiopholis aculeata*. 31. *S. drobachiensis*. 32. *S. franciscanus*. 33. *E. eccentricus*. 34. *C. japonica*. 35 *Strongylocentrotus californicus*. 36. *S. californicus*. 37. *C. chronjhelmii*.

"Amœbocytes with colorless spherules" (Figs. 30–37) are more widely distributed in the Echinoderms than the pigmented ones and are found in the Ophiuroidea, Echinoidea and Holothuroidea. The cells of this type are much more abundant and active in the Holothuroidea than in the other classes. They are also much less stable and disintegrate soon after withdrawal from the body. They are deeply stained by methylene blue-seawater or neutral red-seawater solutions, a reaction which is not characteristic of the "amœbocytes with colorless spherules" in the other classes.

It is to be noted that the size of the spherules is constant for any given amœbocyte, but that there is a variation in the sizes of the spherules of amœbocytes characteristic of different animals. Théel has also remarked upon this and has pointed out that the "amœbocytes with colorless spherules" predominate in the Holothuroidea which he studied. He says: "In the holothurids the white corpuscles are thoroughly predominant, though, there

also, species are to be met with which are characterized by both white and red corpuscles. Thus, for inst., *Labidoplax buskii* makes an exception by lodging cells with hyaline granules together with such ones which contain red pigmented granules, both kinds being nearly equal in number."

The origin and functions of the "amœbocytes with colorless spherules" is problematical. According to Cuenot ('91b) they may arise from leucocytes by the addition of spherules of protein and are hence to be regarded as food carriers.

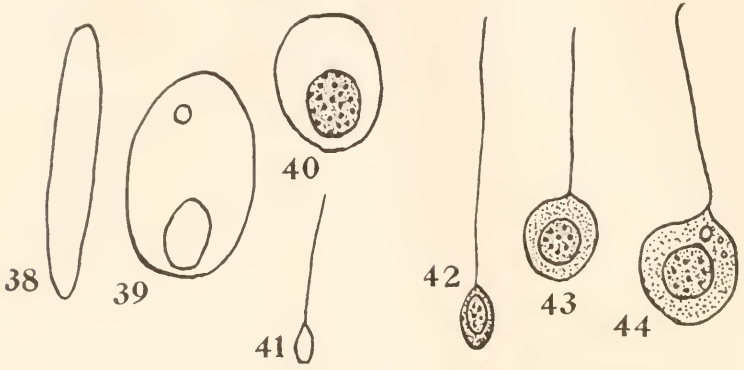



FIG. 38. Hemocyte, *C. japonica*. Side view, cam. luc., × 1300.
FIG. 39. Hemocyte, *C. japonica*. Surface view, cam. luc., × 1300.
FIG. 40. Hemocyte, *C. chronjhelmii*. Surface view, cam. luc., × 1300.
FIG. 41. Vibratile corpuscle, *Stichopus californicus*, cam. luc., × 1300.
FIG. 42. Vibratile corpuscle, *Ophiopholis aculeata*, cam. luc., × 1300.
FIG. 43. Vibratile corpuscle, *Strongylocentrotus drobachiensis*, cam. luc., × 1300.
FIG. 44. Vibratile corpuscle, *S. franciscanus*, cam. luc., × 1300.

### 3. *The Hemocytes.*

Théel ('21) describes a series of "red blood corpuscles" from the perivisceral fluid of the Holothuroidea, excepting the forms with tests, as *Psolus*. In his discussion of these cells, he calls attention to the fact that the content of these corpuscles was first noted by Semper ('68) who suggested that this material was of the nature of hemoglobin. Howell ('85) advanced the same suggestion with regard to the same type of cell in certain species of *Thyone* and *Cucumaria*. Cells of this type were also observed in the Holothuroidea by Cuenot ('91b), Knoll ('93) and Kollman ('08). These cells were described as spherical or elongate ovoid cells with a definite limiting membrane which was elastic, but did

not form pseudopodia. The cytoplasm was of a homogeneous color of the same shade as hemoglobin, and in it was embedded a slightly ovoid nucleus.

In my observations on the genera *Cucumaria* and *Stichopus* I have found these cells to be limited to the *Cucumaria*. For reasons given below I have designated these cells as hemocytes. The hemocytes of *Cucumaria* are flattened, biconvex discs, ovoid in shape. The cell membrane is plastic, but the cells exhibit no amœboid movement and are carried passively in the perivisceral fluid by contractions of the vasa and movements of the body. The cytoplasm is a homogeneous mass of orange-yellow color in which is located a small oval nucleus, eccentrically placed (Figs. 38, 39, 40). In *C. chronjhelmii* the granular content of the nucleus is very clearly apparent (Fig. 40). A mass of these cells presents a crimson appearance comparable to the oxygenated blood of vertebrates. The hemocytes of *C. japonica* (red body wall) are much larger and more numerous than those of *C. chronjhelmii* (white body wall), consequently the deep color of the former may be due in part to this difference in number.

Van der Heyde ('22), although giving no reference to the earlier suggestions that these cells contain hemoglobin, carried on a series of experiments on the content of hemocytes in *Thyone briareus*. He subjected a solution obtained from these cells to the spectroscope and obtained a band characteristic of oxyhemoglobin; upon reduction the solution gave the single band characteristic of hemoglobin and when shaken with air, the double band characteristic of oxyhemoglobin appeared; hemin-like crystals were obtained from the content of the hemocytes. These experiments together with several other chemical tests have led Van der Heyde to conclude that the substance is hemoglobin. As a result of these observations I have designated these cells, types of which also occur in *Cucumaria*, the hemocytes, a term which is briefer and more concise than the appellation red blood cells.

We may ask if there is any reason why oxygen-carrying cells should be present in certain of the Holothuroidea and not in other members of this class or other Echinoderm classes. I have suggested earlier in this paper that oxygen-carrying cells may be regarded as unnecessary in the Asteroidea, Ophiuroidea and Echinoidea because of the relatively low oxygen needs of the

body which can be supplied by free oxygen of the perivisceral fluid. But in the Holothuroidea of the types like *Cucumaria* and *Thyone*, the body is made up of highly contractile muscle elements which may possibly need more oxygen than can readily be supplied by the perivisceral fluid. Consequently, it is suggested that the hemocytes, as oxygen carriers, have appeared in association with the development of the muscular system and greater muscular activity, so that the contractile elements may be supplied. Further evidence for this suggestion is found in the absence of these hemocytes in the Holothuroidea without muscular bodies (*e.g.*, *Psolus*) which are comparable to the test-bodied Echinoidea.

### 4. *The Vibratile Corpuscles.*

The vibratile corpuscles are those cells in the perivisceral fluid which are minute and bear flagella. Cells of this type were observed in *Ophiopholis aculeata* (Fig. 42), *S. drobachiensis* (Fig. 43), *S. franciscanus* (Fig. 44) and *Stichopus californicus* (Fig. 41).

In *O. aculeata* the vibratile corpuscle is very minute (3 microns in diameter). A small peripheral layer of granular cytoplasm encloses a relatively large nucleus. A single long flagellum is present at one end of the cell. The vibratile corpuscles of *Strongylocentrotus* are much larger, each has a relatively smaller nucleus as compared with the amount of cytoplasm and a shorter flagellum than the vibratile corpuscle of *O. aculeata*.

In *S. californicus*, the vibratile corpuscles are very minute (1 micron in diameter) and are colored with a yellowish pigment which obscures the nucleus, if such be present. Since hemocytes are lacking in this species it may be assumed that these cells are oxygen-carrying cells and that the pigment is a hemin compound related to the content of the hemocytes of the other muscular-bodied Holothuroidea.

Cuenot ('02) suggested that the function of the vibratile corpuscles is to keep the fluid content of the perivisceral cavity in motion, thus aiding the ciliated peritoneum in causing a circulation of the fluid.

## III. Summary.

From a comparative study of the cellular elements in the perivisceral fluid of a representative group of Echinoderms found in the vicinity of the Puget Sound Biological Station the following conclusions have been reached:

1. The leucocytes are constant in the perivisceral fluid of the Echinoderms.

2. The leucocytes in all of the Echinoderms studied are phagocytic and thromboblastic and in some species appear to be sclero-blastic and associated with the replacement of resected skeletal areas.

3. Hemocytes (cells with hemoglobin) are found only in certain of the Holothuroidea and are correlated with the development of a highly muscular body.

4. Modifications of the breathing organs have apparently no effect on the cellular contents of the perivisceral fluid, so that no specific oxygen-carrying cells are developed in those forms with a rigid non-muscular body wall despite the limitations of the breathing organs.

5. Of the "amœbocytes with spherules," those with colorless spherules are present in the Ophiuroidea, Echinoidea and Holothuroidea and are predominant in the last class. "Amœbocytes with red spherules" are present in the Echinoidea, where the size and number present is correlated with the depth of color of the body wall.

6. Vibratile corpuscles are present in the Ophiuroidea, Echinoidea and in *Stichopus californicus* alone of the Holothuroidea studied. In the latter species they are pigmented and are regarded as cells which function as do the hemocytes in other muscular-bodied Holothuroidea.

## Acknowledgments.

I wish to take this opportunity to express my thanks to the Ohio Academy of Science for a research grant which aided me in carrying on this investigation.

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# CELL BEHAVIOR IN TISSUE CULTURES.

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This paper deals with certain free wandering cells observed in tissue cultures from the minnow, *Fundulus heteroclitus* and *Fundulus majalis*. The types of cells which became free and isolated from the spreading tissue growths were, chromatophores, amœboid mesenchyme cells and, most abundant of all, certain cells having curious fan shaped projections. These cells proved to be identical with those studied in their association in tissues in cultures by Dr. Dederer ('21). She identified these as mesenchyme cells which in cultures were the means of attachment of the outgrowing sheets of ectodermal cells to the surface of the coverslip. In the work here presented isolated cells were sought as the best objects for the study of cell behavior. The mode of locomotion and the tactile reactions were more especially studied and for the latter work the Barber microdissection apparatus was utilized. The tissues were cultivated in the sea water medium (M. R. Lewis, '16) using, however, in many cases more dilute solutions. Observations were usually made within two days after planting. The work was done at the Marine Biological Laboratory at Woods Hole, Massachusetts during the summers of 1921 and 1922. I wish to acknowledge my indebtedness to Mrs. D. B. Young for the drawings from the stained preparations and to Mr. S. C. Williams for aid in certain of the observations on the rate of motion of cells.

*The Fan Cells*—Among cells of this type an abundant form was that for which I came to use the descriptive term "Canoe cells." When first observed these seemed to be elongate spindle-shaped cells such as indicated by many outlines in Fig. 4. I supposed that there were delicate psuedopedia at either end but upon plotting the direction of the motion of these cells I was surprised to find that they were moving steadily at right angles to the long axis. More careful observations upon living and upon fixed and stained preparations showed the presence of a delicate

film along what proved to be the anterior side of the cell. This film or fan rounded about the ends of the elongate cell, thus giving the canoe-like form (Fig. 1). By use of the microdissection

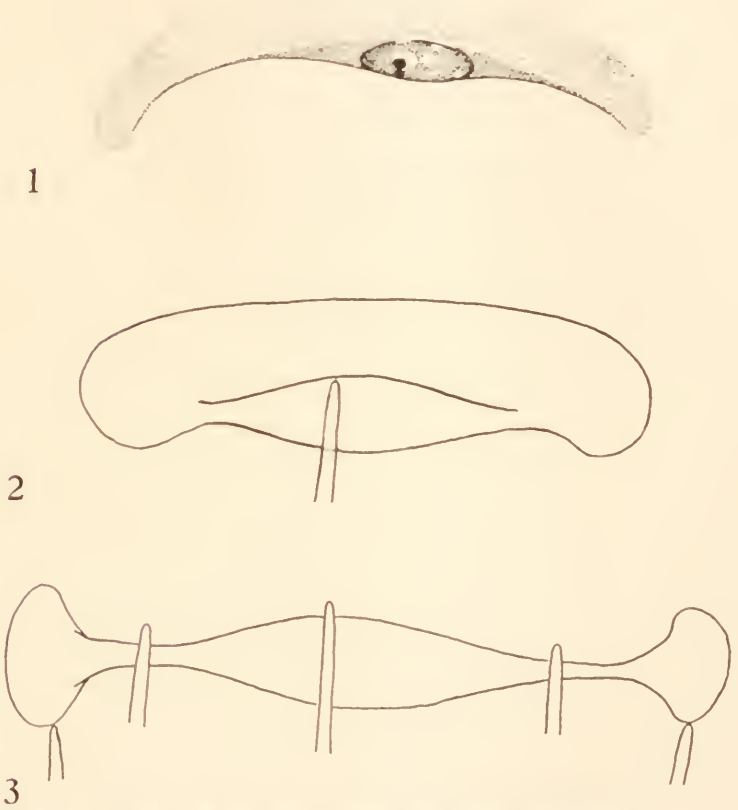


FIG. 1. Typical "Canoe" cell. Drawn from stained preparation.

FIG. 2 and 3. Diagrams of "Canoe" cell (2) and double fanned or bipolar cell (3) showing microdissection needle above body of cell and beneath the cover glass. The fans are firmly attached and the needle can not be pushed between them and the glass.

apparatus the relation of the cell to the cover glass was determined more accurately. It was possible to slide a needle between the more visible spindle shaped part of the cell and the cover glass. The, fan, however, was firmly attached (Fig. 2). The fan was clearly of ectoplasm in the gel state while there was more fluid protoplasm within the body of the cell.

I believe these fans to be the motor organs of the cell. There

seemed always to exist a definite relation between the position of the fan and the direction of motion. The fans were the only portion of the cells in contact with the solid support and there seemed to exist no mechanism for locomotion in a fluid medium without support. Other types of fan cells, as described below, illustrate these points even more clearly than the "Canoe" cells. The rate of motion of the "Canoe" cells was studied by plotting their course with a camera lucida. Fig. 4 shows the history of such a cell. In most cases the outline of the fan could not be observed with the camera in position and an outline of only the body was drawn. However, in positions 1, 12, 21, 28, 30, 32 and 34 the probable form of the fan is indicated by dotted lines. These outlines were based on observations with the prism of the camera removed. At position 8 the cell became attached by a psuedopodium-like projection on the right which may have terminated in a fan. A similar process occurred at positions 24 to 35 during which period a small fan could clearly be observed at the right. At position 27 the cell under observation collided with another cell. The two cells became attached and the newcomer formed an irregular projection at the upper right of the original cell (positions 27 to 36).

The average rate of motion of freely moving cells excluding such cells as proved to be slowing down prior to the death of the cell, was 6.3 microns per minute. The cell shown in Fig. 4 moved at a rate of 5.3 microns from positions 1 to 25. An attempt was made to study the effects of changes of temperature upon the movement of these cells. For this purpose the cultures were studied under the microscope in a warmed box at temperatures varying from 21° centigrade to 42°. Above 40° the cells withdrew their fans and became rounded. Observations were made at constant temperatures and also during an increase of temperature. It soon became apparent that variations in the conditions of individual cells would preclude the possibility of constructing a temperature curve for the rate of locomotion. Cells becoming free from the main growth of tissue moved for a variable period and then became rounded and this condition probably preceeded the death of the cell. A slowing of the rate of motion was apparent before the contraction took place. Also the varying form of the cell and the probable occasional attachment by small sub-

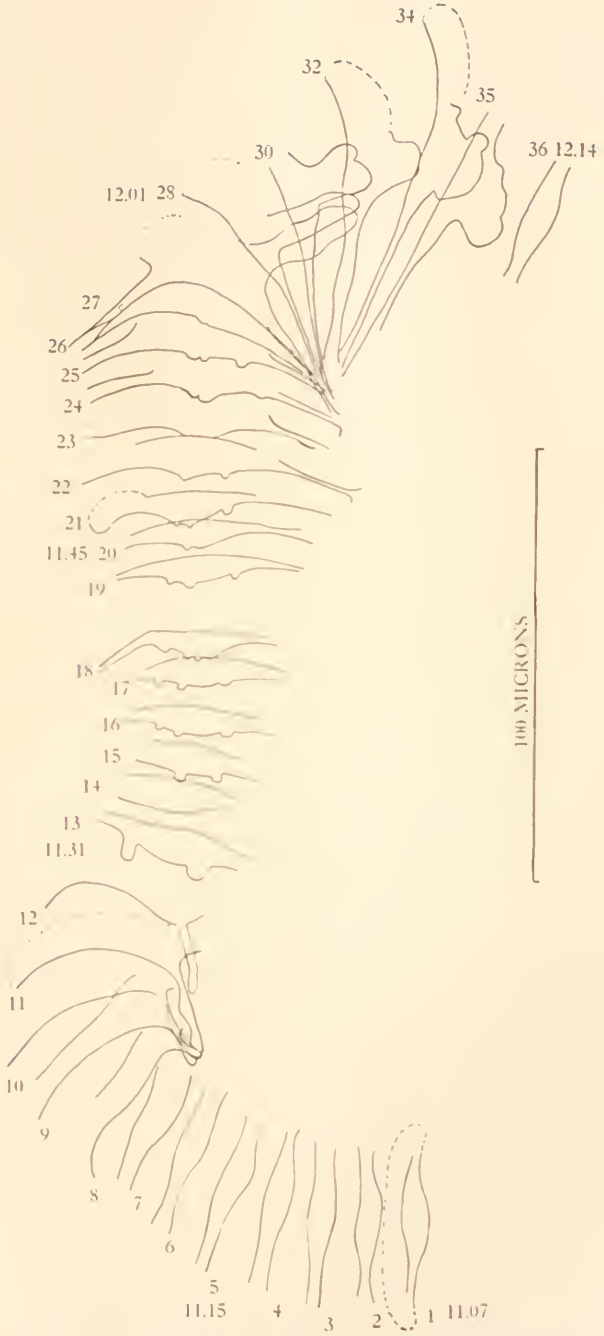


FIG. 4. The history of a "Canoe" cell for one hour and seven minutes at room temperature. The solid lines outline the cell body in so far as it was visible when projected by the camera lucida. The dotted lines show boundary of fans based on observations with the prism of the camera raised.

sidiary fans, as in Fig. 4, which are invisible when making camera drawings, doubtless influence the rate of motion. Nevertheless the results seem to indicate that an increase of temperature causes an increase in the velocity of the movement. The average of all (15) observations at room temperature showed a rate of locomotion of 6.3 microns per minute. The average of all (10) observations at higher temperatures (in most cases varying) was 7.3 microns per minute. The records of greatest speed were 11.5 microns and 10 microns per minute and were attained by cells at the higher temperatures. In the latter case the cell was followed for 39 minutes. The details of these observations are recorded in the tables in the appendix to this paper.

A second form assumed by these cells was that exhibiting two fans (Figs. 5, 6, 7). These fans were usually at opposite poles and under their influence the cell became greatly attenuated. In such cases a micro-dissection needle could be passed between the coverslip and the body of the cell (Fig. 3), but the fans were found to be firmly attached. The cell was thus freely suspended like a hammock between two supports—the fans forming means of attachment and also of extension. Two typical double fanned or bipolar cells are shown in Fig. 5 and 7 which were drawn from stained preparations. In Fig. 6 are shown two cells attached with one fan pulling in a direction not directly opposed to the other.

Another extraordinary mode of motion was observed in which the contractility of the cell functioned as well as the gliding motion of the fan. The history of such a cell is shown in Fig. 9. The account begins at 2.44 P.M. with the cell at position 1 and with a fan at either end. Suddenly a release of the fan occurs and the cell contracts and is thrown into position 2. It again elongates, fans are found at either end—positions 3, 4, 5 and at 3.01 a second contraction occurs, throwing the cell into position 6. The process is repeated four times—positions 6–9, 9–12, 12–13, and 13–18. In each case the cell is elongated by the pulling of the opposed fans. This unusual mode of motion was observed in a culture from a 17-day embryo. This marked contractility of a mesenchyme cell is almost suggestive of the behavior of muscle cells as described by M. R. Lewis ('20) except that these cells

FIGS. 5, 6, 7, 8. Typical fan cells drawn from stained preparations. Figs. 5 and 7 are bipolar or double fanned cells. The limits of the fans may have extended further than indicated. Fig. 6. Fan cells—possibly shortly after cell division. Fig. 8. Two cells apparently fused forming syncytium. Magnification about 1800 times.

contract completely to a spherical form and then gradually expand.

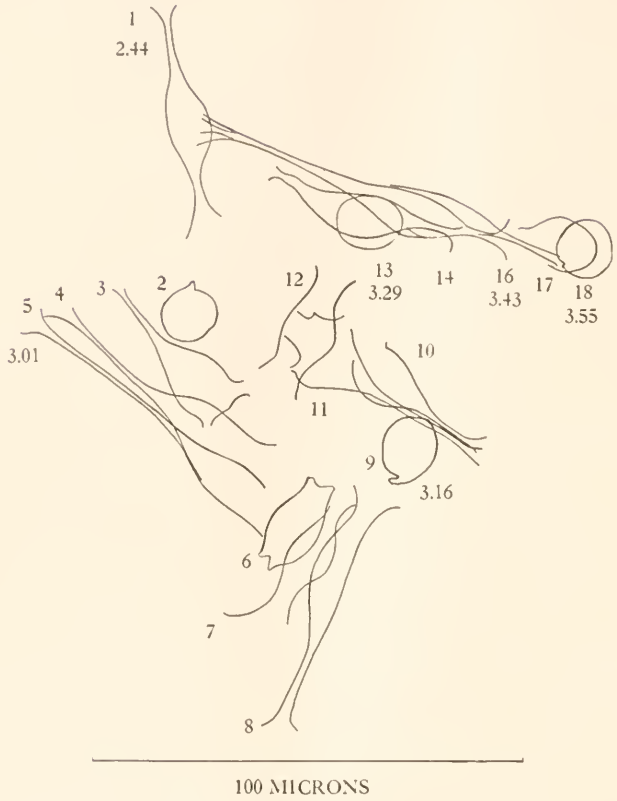


FIG. 9. The history of a bipolar fan cell for one hour and eleven minutes at room temperature of 23°. Movement is by method of alternate expansion and contraction. For details see text.

The cells having fans were found only in cultures showing epithelial growths such as described by M. R. Lewis ('16) and Dederer ('21). I have planted cultures from embryos of various stages but have been unable to obtain this type of growth from those of less than six days of age when developing at the temperature of the running sea water in the laboratory (19° to 22°). The most favorable period for planting and obtaining such growths is shortly before hatching (about 17 days, 19°–22°). Sections of embryos of these later stages reveal a conspicious layer of cells beneath the surface epithelium which are almost completely

wanting in the earlier stages. Dr. Dederer's observations have shown the close relation between epithelial cells and the fan cells and it is then not improbable that this layer contains the fan cells. Moreover she has shown these fan cells to be necessary for the spreading of the epithelial growth by attaching it to the cover glass. Therefore the absence of the epithelium in cultures from the younger embryos may be due to the absence of this sub-epithelial layer with its included fan cells.

In a few cultures other unpigmented cells of presumably mesenchymal origin and of exceedingly irregular form were noted. Their mode of motion seemed typically amœboid.

*Tactile Reactions.*—The tactile reactions of various types of cells were studied. For this purpose the cells were touched with a delicate glass needle moved by a Barber micro-dissection apparatus. If the body of a double fanned cell such as that shown in Fig. 3 is sharply stimulated the fans are released and the whole cell contracts, becoming spherical. A fan may be pried loose and a similar reaction ensues. The contraction seems in part due to an elastic tension of the cell. I have not found it possible to stimulate a cell by touching or even partially mutilating a small portion of the fan. The ectoplasm does not seem to conduct a stimulus. In some cases a delicate stimulus near the boundary of a fan and the cell stalk of a greatly elongated cell will cause complete contraction. The material of the fan seems to flow together, making a ball of protoplasm which is carried along with the stalk to the center of the cell.

I have tried a few experiments to determine the chemotactic response of such tissue cells. A cloud of methylene blue injected by a micro-pipette will cause the contraction of these isolated cells. I have not succeeded in obtaining any more definite response such as a change in the direction of motion.

Previous writers, Bancroft ('12), Stockard ('15) and Newmann ('18) have described two types of chromatophores in the *Fundulus* embryo. These are black chromatophores or melanophres and the brown (or red) chromatophores. Bancroft ('12) has described a third type which also appeared in these tissue cultures. These were yellow and smaller than the other types and showed few or no pseudopodia. As a group, the chromatophores are but slightly responsive to tactile stimulation. If a needle is pushed against

a pseudopod with sufficient force to slightly indent the ectoplasm, the pseudopod may slowly withdraw. A brownian movement of pigment granules is frequently initiated. Of these cells the yellow chromatophores are most responsive, the red are less responsive and the melanophores are almost completely inert to tactile stimulation.

All cells studied seem to show less variety of adaptive response than the amœba. Thus the only reaction that I have observed is contraction either of the whole or a portion of a cell.

I have previously referred (Goodrich, '22) to the motion of the fan cells as non-amœboid. The characteristic streaming of protoplasm which we associate with the amœba is certainly not present. The phenomenon seems more akin to the movement of diatoms. It is, however, not impossible that this gliding motion may be a factor in the locomotion of many unicellular organisms. Schaefer ('20) in his discussion of amœboid movement has called attention to the importance of a surface film of streaming protoplasm external to the ectoplasm and wholly distinct from the familiar streaming of the endoplasm. This film can be observed only indirectly as it carries particles that become entangled in it. Schaefer states (page 106) that "the surface film in amœbas is powerful enough to enable them to move by it." In this case it causes a backward motion. It is also probable (see Schaefer, '20, for discussion) that such a surface film is important in the movement of diatoms, *Oscillitoria* and even of Gregarines. No adequate explanation has been offered for the motion of this surface film. I have not been able to detect the presence of such a moving film in the fans of the cells studied in this paper although cells have been observed in media containing a suspension of carbon granules. Yet the delicacy of the fan is such as to make the test seem inadequate and it is not impossible that such a mechanism may exist. If so we may have some clue to the motion of many cells in development and in regeneration. Moreover this mode of motion seems allied to the power of adhesion of cells. The cells here described are attached to the cover glass by means of the fans. Dr. W. H. Lewis ('22) has raised the question as to why tissue cells adhere in an organism. The mechanics of the gliding motion seems to involve this power of adhesion and thus the two problems may be related.

SUMMARY.

1. Certain isolated cells from tissue cultures of *Fundulus* embryos have been described.

2. These cells posess fan-shaped films which are adherent to the cover glass.

3. These films are the motor organs of the cells by means or which they glide on the under surface of the cover glass.

4. The tactile reactions of these cells and of the chromatophores are described.

5. The relation of this type of cell movement to amœboid movement is discussed.

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## APPENDIX.

### TABLE I.

RECORD OF OBSERVATIONS ON RATE OF MOVEMENT OF CELLS AT ROOM TEMPERATURE.

| Reference Number. | Average Rate in Microns per Minute. | Temperature in Degrees Centigrade. | Duration of Observations in Minutes |
|---|---|---|---|
| 1 | 3.4 | 24.5 | 41 |
| 2 | 3.3 | 24.5 | 42 |
| 3 | 7.6 | 24 | 16 |
| 4 | 4. | 24. | 33 |
| 5 | 4.1 | 24. | 19 |
| 6 | 5.9 | 24. | 11 |
| 7 | 8.1 | 22.5 | 25 |
| 8 | 8.9 | 22. | 15 |
| 9 | 8.2 | 22. | 11.5 |
| 10 | 7. | 22. | 8.5 |
| 11 | 6.1 | 22. | 9. |
| 12 | 8. | 21.5 | 20 |
| 13 | 7.7 | 21.5 | 13 |
| 14 | 8. | 21.5 | 7.5 |
| 15 | 5.3 | 22. | 48. |

### TABLE II.

RECORD OF OBSERVATIONS OF RATE OF MOTION OF CELLS AT HIGHER TEMPERATURES.

The temperature was in most cases gradually increased as indicated.

| Reference Number. | Average Rate in Microns per Minute. | Temperature in Degrees Centigrade. | Duration of Observations in Minutes. |
|---|---|---|---|
| 16 | 8.3 | 29.5–37 | 25 |
| 17 | 11.5 | 27 –33.8 | 20 |
| 18 | 8.3 | 33.8–36.5 | 25 |
| 19 | 3.4 | 27–35 | 44 |
| 20 | 9.1 | 35 | 7 |
| 21 | 5. | 27–30 | 12 |
| 22 | 5.8 | 28–31 | 8 |
| 23 | 6.4 | 27–31 | 13 |
| 24 | 5.1 | 27–29 | 10 |
| 25 | 10. | 33–35 | 39 |

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# BIOLOGICAL BULLETIN

## STUDIES ON THE SECONDARY SEXUAL CHARACTERS OF CRAYFISHES.

### I. Male Secondary Sexual Characters in Females of *Cambarus propinquus*.

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During the early spring of 1922 the writer found five specimens of *Cambarus propinquus* which had both male and female secondary sexual characters. Two of the specimens were destroyed in dissecting the internal organs, in examining the annulus for sperm and in separating the appendages preparatory to drawing them. A review of the literature revealed that the condition is extremely rare. Many thousands of specimens have come under the close scrutiny of Faxon, Hagen, Hay, Ortmann and others and only thirteen cases in the genus *Cambarus* have been recorded in which male and female secondary sexual characters have been found to exist in the same individuals. The finding of the five specimens seems all the more remarkable because all were taken within a short distance of each other in Turtle Creek near Beloit, Wisconsin on a single field trip. About two hundred specimens were taken on the trip so that the seeming hermaphroditic ones constituted about 2.5 per cent. of the entire catch.

Diligent search was made in the same locality during the late spring with the result that three more similar specimens belonging to the same species were found.[1] The eight specimens are referred to in the descriptions as numbers 1 to 8 inclusive.

The search was continued in 1923 resulting in the capture of four females in the same locality in Turtle Creek. All showed

[1] Six of the eight specimens described have been deposited in the Carnegie Museum at Pittsburgh.

definite secondary sexual characters of the male. All four were carrying eggs or embryos attached to the abdomen, making it certain that they were fully functional females in spite of the presence of male secondary sexual characters. These four will be referred to in the descriptions as numbers 9, 10, 11 and 12. The brood of one of the specimens was successfully reared. Eight months after hatching twelve members of the brood were still thriving and it is hoped that the effort to maintain and breed them under laboratory conditions will be successful.

Later in the summer of 1923 one hundred females were taken from South Kinnikinick Creek a tributary of the Rock River fifteen miles south of Beloit. Among these were found seven having the partial development of male secondary sexual characters. These seven will be referred to as numbers 13, 14, 15, 16, 17, 18 and 19.

Other streams in the vicinity of Beloit have been searched and a considerable number of abnormalities in the secondary sexual characters have been found. In no female individual, however, has there been found any male secondary sexual character.

The fact that this simultaneous occurrence of male and female secondary sexual characters in the same individual has been found only thirteen times in the entire genus *Cambarus* makes the discovery of nineteen specimens in a single species within a limited area well worth investigating.

This same condition has been found to exist in southern Wisconsin to a still greater degree in *Cambarus virilis*. Eighty-eight per cent. of all the females taken in Delavan Lake show additional, male secondary sexual characters.

It seems likely that the conditions causing the high rate of peculiarities is a local one and it is proposed to go into the matter thoroughly and to publish results whenever a body of new data has been collected or whenever any conclusions have been reached.

## Review of Literature.

The first described instance of a truly hermaphroditic decapod crustacean is that of a lobster (*Homaris vulgaris*) which had normal external genitalia and internal organs of the male on the left side and those of a female on the right side. The specimen

was described by F. Nicholls in 1730. Another specimen has been described in the Canadian Naturalist, Vol. XXXIII, No. 2.

Rosseau and Desmarest described several cases in *Astacus fluviatilis* of branched oviducts opening on the third and upon the fourth walking legs.

Von Martens described three specimens of *Cheraps preisii*, an Australian crayfish, in which there were openings at the base of the third walking legs but no oviducts and no ovaries. The specimens were normal males except for this peculiarity.

Von Ihring, Lönnberg, Faxon and Hay working upon various species of Parastacus, a genus found in South America, found that there were three constant conditions of the genital glands, ducts and external genitalia. Two of these conditions pointed toward a state of partial hermaphroditism. In some species there were single genital glands, either spermaries or ovaries, and two sets of genital ducts and genital openings. Only one of the sets of tubes was functional, however. In other species a single genital gland and two sets of genital tubes existed but there were no genital openings for the non-functional tubes. In still another species there was no evidence either of extra genital tubes or of extra genital openings. The normal condition in the first two subdivisions of this genus was, therefore, one of partial hermaphroditism.

Faxon (1885) found four cases in the genus *Cambarus* which gave some evidence of hermaphroditism.

One specimen of *Cambarus propinquus sanborni*, 60 mm. long, had the claws of a female, the abdominal appendages of a female and a well-formed annulus but no female genital openings at the base of the third walking legs. Instead, it had the external genital openings of a male at the base of the fifth walking legs. Dissection demonstrated the presence of a well-developed ovary so the specimen was evidently a female.

The second case was also one of *Cambarus propinquus sanborni*, 38 mm. long. The specimen was female in every respect except that the first abdominal appendages were like those of the male.

The third case was a specimen of *Cambarus diogenes*, 84 mm. long, in which the external genitalia were those of a female with the exception of the first abdominal appendages which were

almost as large as those of a male but were lacking the characteristic, recurved hooks.

The fourth specimen, a female of Cambarus *propinquus*, 72 mm. long, was normal with the exception of the first abdominal appendages. These were modified so as to be almost identical with but slightly smaller than those of the male.

Hay (1905) in examining the collection of crayfishes in the U. S. National Museum which had been made by the U. S. Fish Commission and by himself found four additional specimens which showed evidence of hermaphroditism.

In the first specimen, *Cambarus spinosus*, the organs internally and externally were those of a female with the exception of the annulus ventralis which was rather small. In addition there were the following male secondary sexual characters: hooks on the base of the third walking legs, abdominal appendages modified as in the male but slightly undersized, genital papilla at the base of the fifth walking legs, but no genital pores.

The second specimen, also *Cambarus spinosus*, was identical with the first with the exception of the first abdominal appendages which were shorter.

The third case, a specimen of *Cambarus propinquus*, 53 mm. long, displayed a preponderance of male secondary sexual characters on the left side and of female characters on the right side. The right side had the usual genital pore of the female at the base of the third walking leg, a small abnormal appendage on the first abdominal segment and the usual second abdominal appendage of the female. The annulus ventralis was also well developed. On the left side there was no female genital opening at the base of the third walking leg but on this third leg there was the copulatory hook normally found in the male. The first and second abdominal appendages of the left side were those of a normal male and at the base of the fifth walking leg there was the genital pore of the normal male. Nothing of the internal structure could be made out.

The fourth case appeared in a specimen of *Cambarus affinis*, 106 mm. long. Externally it had all the characters of a male except that it had no opening for the sperm duct at the base of the left fifth walking leg. It did have, however, the genital openings

of the female at the base of the third walking legs. A dissection revealed that there were fully developed ovaries and oviducts on both sides and a small testis with a sperm duct on the right side.

Hay concludes that in the genus *Cambarus* hermaphroditic individuals are females "which owing to some ambiguity of the formative cells in the embryo have developed to a greater or less degree the characters of the opposite sex. The condition is a very rare one and is usually shown in the external organs only". . . . "Hermaphroditism is as uncommon in *Astacus* as in *Cambarus*. Among the Parastacidæ the condition of apparent hermaphroditism seems to be established in the genus *Parastacus*. It may also be found in *Cambarus* but is rare or altogether wanting in other genera."

Ortman (1905) described five additional cases in the genus *Cambarus*, four collected by himself and one by E. B. Williamson.

His first case was one of *Cambarus obscurus* which had as female secondary sexual characters the female type of claw, a definite but small annulus and no hooks on the third walking legs. The specimen had, however, the genital openings of the male at the base of the fifth walking legs and the first and second abdominal appendages, clearly of the male type.

The second specimen was also one of *Cambarus obscurus* which had not only the annulus and the genital openings of the female but also copulatory hooks on the third leg (a male characteristic). The abdominal appendages were abnormal but full-sized and male-like. The specimen was interpreted as being a female with the full but not specific development of male organs.

In the third case the specimen (*Cambarus bartoni*) was a functional female, 71 mm. long with eggs attached to the abdomen. It showed the full equipment of secondary sexual characters of the female and in addition a pair of hooks at the base of the third walking legs.

The fourth individual, also a specimen of *Cambarus bartoni* (48 mm. long), had female claws, a well-developed annulus and the second abdominal appendages of the female. The usual hooks at the base of the third walking kegs were lacking. The male secondary sexual characters were shown in the genital openings at the base of the fifth walking legs and in the full development of the male first abdominal appendages.

The specimen collected by Williamson was one of *Cambarus rusticus*. The first abdominal appendages were not fully developed but were clearly of the male type while all the other secondary sexual characters were of the female type.

## Description of New Cases.

*Specimens 1, 2, 3, 4 and 5.* (Fig. 5.) These five specimens are identical in their peculiarities and it will therefore suffice to describe one of them and to give the lengths of the other four. The length of the specimen described is 48 mm. The lengths of the others are 45, 47, 49 and 52 mm. respectively. In the specimen 48 mm. long the secondary sexual characters are typically female but there are the following additional characters: copulatory hooks are present at the base of the third walking legs; the first abdominal appendages are modified to resemble somewhat those of the male of the first form, being shorter, however, (8 mm. in length) with the tips undivided and curved toward the median line (Fig. 10); the second abdominal appendages are those of a normal male (Fig. 10). A dissection of the internal organs has been made and ovaries and oviducts have been found. The eggs are full sized and would normally have been laid within two months. The ovaries are heavily parasitized by the embryos of a flatworm [1] to such an extent that many of the eggs are entirely replaced by the encysted embryos. No trace of a testis or of a vas deferens is present.

*Specimen number* 6 is 42 mm. long. It differs from numbers 1, 2, 3, 4, and 5 only in the degree of development of the first abdominal appendages. The right member of the pair is in all respects like the homologous appendage of the normal male of the second form. The left member of the pair is slightly shorter and the tip is bent toward the median line.

*Specimen number 7* is 54 mm. in length and is typically female in all its secondary sexual characters except in the case of the two abdominal appendages. The first abdominal appendages are modified like those of cases 1, 2, 3, 4 and 5 and are of the same size. The second abdominal appendages differ only slightly from those of the female, being a little stronger and the inner

[1] The writer is indebted to Professor A. S. Pearse of the University of Wisconsin for identifying the parasites as *Microphallus opacus* Ward.

ramus a little thicker. There is no trace of the triangular shoulder which occurs regularly on this appendage in the normal male. This specimen has been dissected and as in the previous cases there have been found well developed ova. This case is unquestionably one of a female with the partial development of male secondary sexual characters. The male characters are not so strongly developed as in the previous cases, the hooks on the third walking legs being absent and the second abdominal appendages being modified but little from the usual female type.

*Specimen number 8* is 47 mm. long. It is without doubt a female, having a normal annulus ventralis and oviducal openings at the base of the third walking legs. The first abdominal appendages are very peculiar, however, being unlike on the two sides. The right one is a normal diminutive female appendage. The left one (Fig. 11) appears to have grown as a bifurcated female appendage but there has grown out of it at the junction of the basal and distal parts an appendage somewhat similar to and a little smaller than the normal first abdominal appendage of the second form male. The proximal half of the appendage is quite slender. The second abdominal appendages, both in size and general form resemble the normal female appendages but a distinct triangular shoulder like that found in the male is present upon the endopodite (Fig. 14).

*Specimen number 9* was bearing well-developed eggs upon the abdomen when taken so there was no question as to the sex. It is 67 mm. in length. The oviducal openings and the annulus ventralis are those of a normal female but the following male structures are also present: copulatory hooks on the third walking legs; first abdominal appendages much like those of the normal first form male; second abdominal appendages like those of the male in form but a little smaller and with the triangular shoulder on the endopodite not so well developed (Fig. 4).

*Specimen number 10*, 55 mm. long, is unusual in that it possesses an extra oviducal pore at the base of the left fourth walking leg in addition to a rather complete complement of both male and female secondary sexual characters (Figs. 3, 9 and 14). The annulus ventralis and oviducal pores at the base of the third walking legs are normal female characters. An extra oviducal opening is present on the left fourth walking leg but it is

small and has no oviduct attached to it. The openings of the sperm ducts at the base of the fifth walking legs are in the same position as those of the normal male but are smaller and are not so prominent. The first abdominal appendages are shaped much like those of the normal, second form male but they are a little smaller and are more slender in the basal third. The second abdominal appendages are like those of the normal female with the addition of a marked triangular shoulder upon the endopodite, indicating a tendency on the part of the appendage to develope specific male characteristics.

This specimen was dissected and it was found that the internal structures were those of a female. The extra oviducal opening and the openings at the base of the fifth walking legs had no internal tubes connected with them. There was no trace of a spermary.

*Specimen number 11* is 62 mm. long and is identical in its external peculiarities with specimen number 9. Like specimen number 9 also, it was bearing young embryos upon the swimmerets when taken and was of course a functional female. With the eggs so recently discharged it would be expected that the ovary would be depleted and small. A large genital gland has been found, however, which is irregular in shape and seems to be made up of unlike parts. It is possible that the structure may prove to be an ovo-testis.

*Specimen number 12*, 53 mm. long, when taken was bearing embryos which were in an advanced stage. These embryos were reared in the laboratory and forty-nine of the sixty reached a stage in which the secondary sexual characters had become distinguishable. All the embryos were female and all were normal. As in specimen number 11 a large irregular genital gland has been found but there are no ducts except the normal oviducts. Externally this specimen is with the exception of its size almost identical with specimen 11.

*Specimens number 13 to 19 inclusive* (Fig. 6) are alike in their peculiarities, all being normal females as evinced by their internal and external organs but bearing in addition one male secondary sexual character, namely a hook on the left third walking leg. The lengths of the specimens number 13 to 19 are respectively 63 mm., 47 mm., 49 mm., 47 mm., 42 mm., 45 mm., and 50mm.

## Discussion.

The occurrence of both male and female secondary sexual characters in the same individual is so rare and sporadic that it has seemed futile to speculate as to its cause. However, when nineteen instances are found in a limited area within two years it would seem that a close study of conditions might yield results. Two possible explanations suggest themselves.

I. The gonads may have been damaged by parasites so that the possible control of the development of the secondary sexual characters would be lacking. Parasites[2] were found, indeed, in great numbers in both males and in females and there can be no doubt that they did extensive damage to the tissues of the crayfishes. Ovaries, testes, alimentary canals, green glands and livers were often heavily infested and partially destroyed. The seeming effect of parasitism of the gonads upon the development of the secondary sexual characters has been worked out in great detail by Smith (1910). This author found great numbers of the Spider Crab (*Inachus*) so parasitized by *Cirrepede Sacculina* that the ovaries and spermaries of *Inachus* were frequently destroyed. The findings with regard to the effect upon the secondary sexual characters and his conclusions may be found in the following statement: "Whereas the males under the influence of the parasite, were capable of assuming all the female secondary sexual characters and under certain conditions might even develope ova in their testes, the infected females on the other hand, although the ovaries in some case completely disappeared, never approached to the male primary or secondary sexual characters in the slightest degree." ". . . The presence of *Sacculina* caused young females under 13 mm. in carapace length to assume prematurely the adult type of abdomen and abdominal appendage." ". . . it would appear that the male was a potential hermaphrodite and the female purely female."

If the irregularities described in *Cambarus propinquus* are due to parasitism it might be expected that the number of irregularities would be somewhat proportional to the number of heavily parasitized individuals. This, however, does not prove to be the case for parasitism is quite common and irregular secondary sexual characters are rare. Again, if parasitism causes these

irregularities some principle other than that found by Smith for *Inachus* must be sought in *Cambarus propinquus* to explain the relations between the gonads and the secondary sexual characters for in *Inachus* "the male is potentially hermaphroditic and the female purely female" while in *Cambarus* in the instances cited the male shows no tendency to develope secondary sexual characters of the female although the female in all the cases described has developed more or less fully the secondary sexual characters of the male.

II. In *Parastacus* the presence of both male and female secondary sexual sharacters in the same individual is a fixed condition which is transmitted from one generation to another without change. The question arises as to whether or not the condition may not be transmitted in *Cambarus* as in *Parastacus* although much less firmly fixed in *Cambarus* than in *Parastacus*. The finding of a considerable number of specimens within a very limited region would tend to strengthen the view that the condition was somewhat constant in *Cambarus* and was being transmitted. The females seem to be unaffected by the presence of the male secondary sexual characters and they are certainly sexually functional as shown by the fact that all such specimens taken during the breeding season were bearing eggs or embryos. One of these broods was reared and about fifty out of the sixty embryos were brought to a stage in maturity where their sex could be determined. It is rather suprising that every one of those brought to maturity was a female. Twenty-one were carried without mishap to a stage when the secondary sexual characters were fully developed and all were normal, showing no traces of the conditions which existed in the mother.

It is noteworthy that specimens taken within a short distance of each other at the same time should closely resemble each other in their deviation from the normal. The specimens taken from Turtle Creek in 1922 show the peculiarity in the first abdominal appendages illustrated in figure 10. Those taken from Turtle Creek in 1923 possess first abdominal appendages like those illustrated in figures 9 and 11 while the seven taken from South Kinnikinick Creek are all alike in having a single male character, one hook on the left third walking leg. This would indicate that the individuals resembling each other might have been members of the same brood.

To say that the irregularities in the secondary sexual characters may be transmitted is not to answer the question of their origin. It would imply, however, that the cause producing the effect was deeply seated in the germ cells and was not brought about by a temporary, somatic influence.

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## EXPLANATION OF PLATES.

### Plate I.

Fig. 1. Diagram showing the three basal segments of the five walking legs and the first two abdominal appendages with the second pair of abdominal appendages turned back. Normal male. I., II., III., IV., V., walking legs; *AP1* and *AP2*, first and second abdominal appendages; *H*, copulatory hooks on third walking legs; *S*, external openings of sperm ducts at base of the fifth walking legs.

Fig. 2. Diagram illustrating the three basal segments of the five walking legs and the first two abdominal appendages with the second appendage turned back. Normal female. *O*, oviducal openings at the base of the third walking legs; *AV*, annulus ventralis. Other explanations as in Fig. 1.

Fig. 3. Diagram illustrating the three basal segments of the five walking legs, the first two abdominal appendages and the peculiar features described in specimen number 10. Structures may be identified by the explanations in Figs. 1 and 2.

Fig. 4. Diagram of three basal segments of walking legs and first two abdominal appendages with other features peculiar to specimen number 9.

Fig. 5. Diagram of basal segments of walking legs and first two abdominal appendages with other secondary sexual characters shown in specimens 1 to 5 inclusive.

Fig. 6. Diagram to illustrate the condition of the secondary sexual characters in specimens 13 to 19 inclusive.

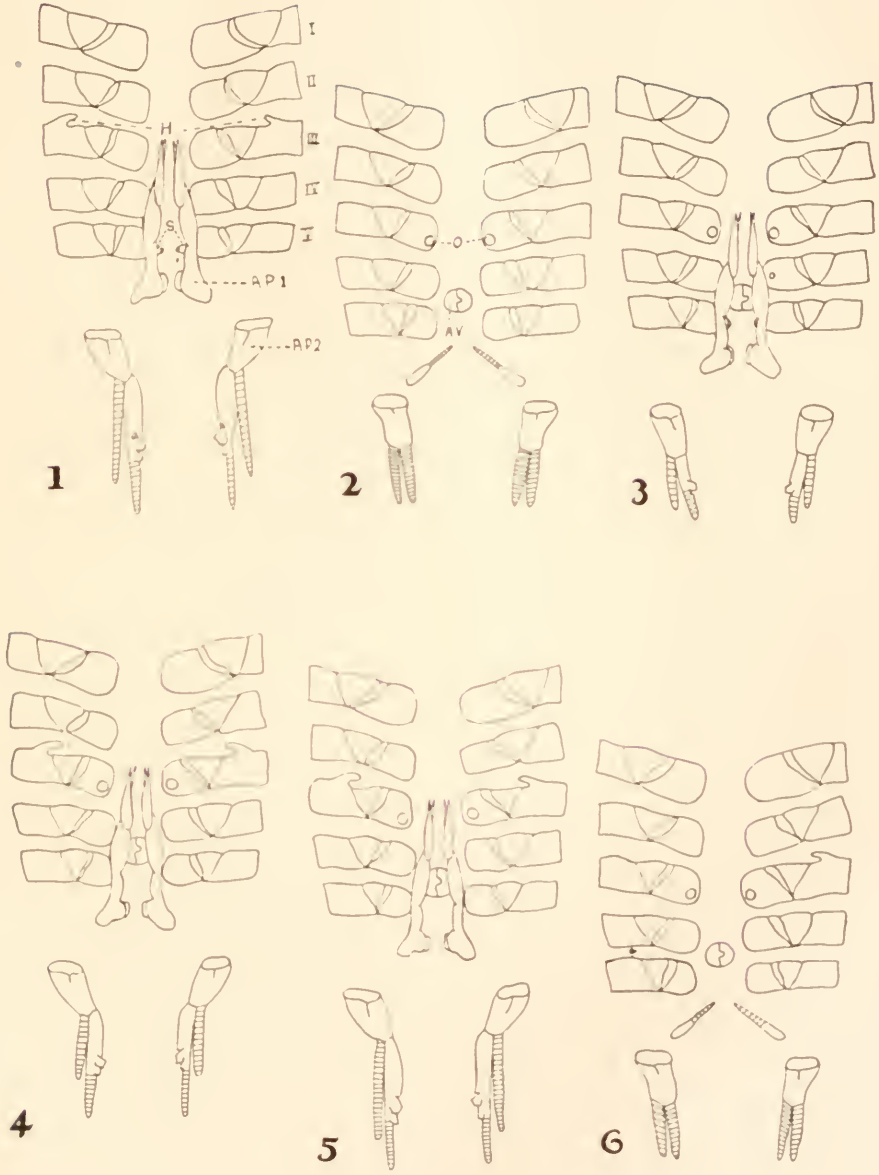


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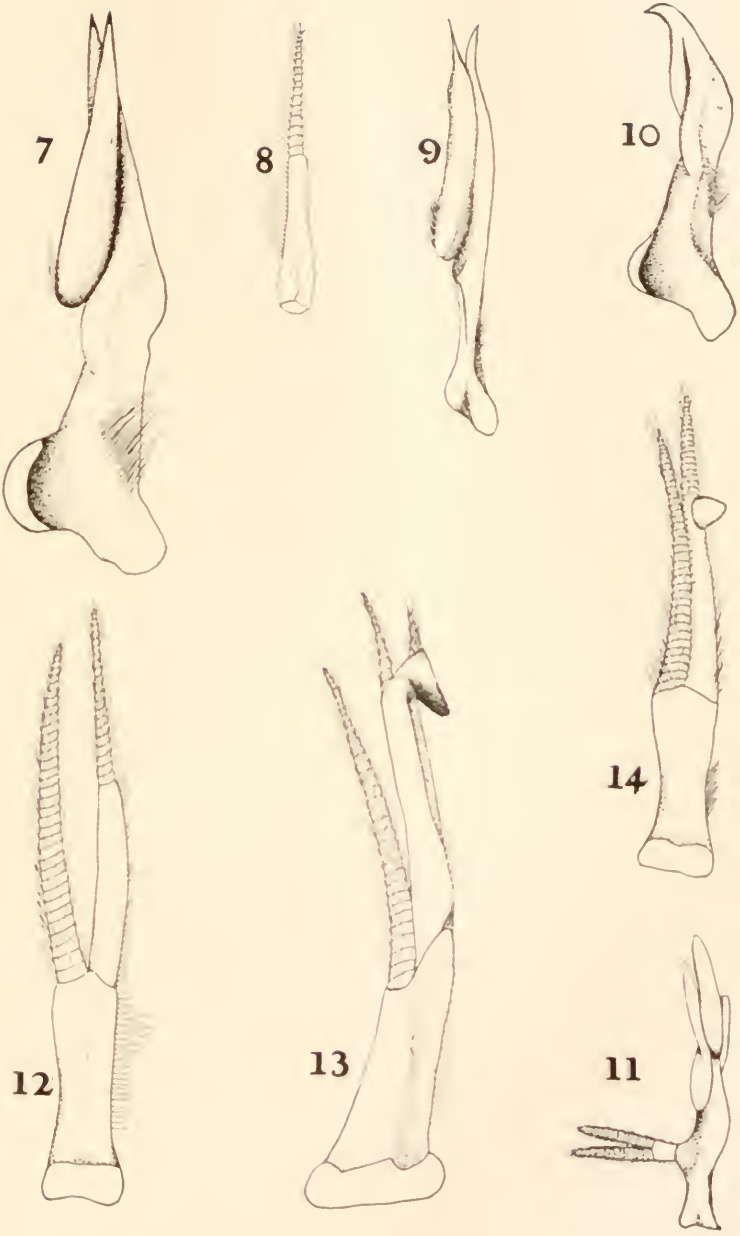
PLATE II.

FIG. 7. Left first abdominal appendage of normal male. × 7.5.
FIG. 8. Left first abdominal appendage of normal female. × 7.5.
FIG. 9. Left first abdominal appendage of specimen number 9. × 7.5.
FIG. 10. Left first abdominal appendage of specimens 1 to 5. × 7.5.
FIG. 11. Left first abdominal appendage of specimen number 8. × 7.5.
FIG. 12. Right second abdominal appendage of normal female. × 7.5.
FIG. 13. Right second abdominal appendage of normal male. × 7.5.
FIG. 14. Right second abdominal appendage of specimens number 8 and number 10. × 7.5.




C. L. TURNER.

# THE SURFACE TENSION THEORY OF MEMBRANE ELEVATION.

L. V. HEILBRUNN.

When a sea-urchin egg is inseminated it lifts off a membrane from its surface. A similar phenomenon can be produced by various reagents. Many explanations have been advanced to account for it. Some years ago, I showed that all substances which produce membrane elevation are those which might be expected to produce a lowered surface tension (1). Also, in general, substances which lower surface tension markedly, produce membrane elevation. And so I advanced the view that surface tension lowering was in all cases related to membrane elevation, and I offered a physical explanation of the process based on such a lowering of surface tension. The idea was new, although Traube (2) had previously shown that when substances of a chemical series are compared, those with lower surface tension are relatively more effective in producing membrane elevation. Traube thought the process involved a secretion on return to sea-water.

The surface tension theory of membrane elevation was accepted by some workers (3) but not by others. Garrey (4) in a review of the literature states that the theory demands the existence of a membrane on the uninseminated egg. For this Garrey finds no evidence, and he cites Moore's (5) repetition of Ziegler's (6) and the Hertwigs' (7) experiment with broken-up eggs as an argument against such a membrane. The evidence for a pre-existent membrane is abundantly supplied in my 1915 paper, to which Garrey does not refer.

In a paper published recently, Just (8) claims that hypertonic solutions of sodium chloride in sea-water, which do not lower surface tension, nevertheless produce membrane elevation.[1]

[1] As a matter of fact hypertonic solutions of NaCl in sea-water do secondarily produce lowered surface tension, for they cause membrane swelling and this produces lowered surface tension.

In my 1915 paper, I pointed out that the earlier descriptions of cortical change in the sea-urchin egg had failed to distinguish between two types of cortical change. The normal change at fertilization is a membrane elevation. Many reagents produce this change, others however produce a swelling of the membrane. The two types of change, although fundamentally quite different, are not easy to distinguish morphologically, partly because the surface of the egg is not especially favorable for microscopic observation. I therefore proposed various criteria to distinguish them. Thus elevated membranes collapse in albumen solutions, swollen membranes do not. Other criteria are easily established. When eggs with elevated membranes are crushed, they flatten and obliterate the perivitelline space. Eggs with swollen membranes have little or no perivitelline space, and when they are crushed, the thick membranes remain around them as before.

Just tried none of these criteria. The membranes he describes in his paper seemingly lack a perivitelline space, for he notes that on return to sea-water from the hypertonic solution, the perivitelline space is not obliterated. If the membranes were separated off as Just believes, and if there were a perivitelline space, one would think that this space would be obliterated by the osmotic expansion of the egg on return to ordinary sea-water.

As soon as an opportunity was afforded, I repeated Just's observations. With the concentrations he used (20–24 per cent. 2½ *M* NaCl in sea-water), no membrane elevation or separation could be observed. In such solutions the membrane could be seen slowly to expand and swell. There was no evidence at all of a sudden movement of the membrane such as occurs when the membrane is elevated. Tested with albumen solutions the membranes did not collapse. When the eggs were compressed the egg contents did not expand to the outer limits of the membrane. The membranes produced by solutions of sodium chloride in sea-water, or on return from such solutions to sea-water, were certainly swollen and not elevated. In the light of this evidence it appears that the argument advanced by Just is not valid. *It still remains true that all substances which produce typical membrane elevation do in every instance cause a lowering of surface tension.*

It should perhaps be pointed out that in cases of extreme coagulation of the protoplasm, the contents of the egg may sometimes be made to shrink away from the vitelline membrane. No good case of this is known for the sea-urchin egg, although some shrinkage apparently occurs after prolonged heat coagulation. In the *Cumingia* egg, which has a much stiffer membrane than the sea-urchin egg, a membrane which does not normally become elevated, some 1916 experiments showed that the protoplasm would shrink away from the vitelline membrane when the egg contents had been thoroughly coagulated after several hours exposure to hypertonic solutions. Such slow coagulative shrinkages are scarcely to be confused with true membrane elevation, although in the *Cumingia* egg under certain conditions the membrane may become stretched away from the egg after it is released.

Just makes one other point against the surface tension theory. He states that in "the eggs of *Arbacia* and *Echinarachnius* any competent observer can see that membrane separation following insemination is no mere surface tension effect." This is not a very serious argument. The surface tension theory claims only that the lowered surface tension is the cause which underlies the process, not that all the details which follow the initiation of the process are surface tension phenomena. And anyway how can one decide a physical problem by mere visual observation?

Just, apparently, has attempted to do this. The lifting off of the membrane is a physical process and demands a physical explanation. Just's nearest approach to this is his description of membrane elevation as involving a "wave of negativity" (9). But he does not use the word in any known physical sense.

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BIOLOGICAL LABORATORY WOODS HOLE

# THE INDEPENDENT DIFFERENTIATION OF ISOLATED CHICK PRIMORDIA IN CHORIO-ALLANTOIC GRAFTS.

## I. The Eye, Nasal Region, Otic Region, and Mesencephalon.

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### I. Introduction.

The study of the power of independent growth and differentiation of embryonic primordia (self-differentiation, Roux) has been pursued by various methods. The experiments on the development of isolated blastomeres, inaugurated by Roux's study of the frog's egg, have contributed greatly to our knowledge of the localization of potentialities in cleavage stages. More recently, investigation of growth and differentiation of embryonic tissues has been carried on by other methods. The classical experiments of Harrison (1907 *a*, 1910) on the embryonic nerve cells of amphibia in tissue culture, followed by numerous studies of other embryonic cells by Harrison (1912), Lewis, M. R. and W. H. (1911 *a*, *b*, etc.), and others, furnish good examples of the specificity and the degree of the independent power of differentiation of the isolated cells of various embryonic organs. Harrison (1907 *b*) and others have also made studies of the independent power of growth and differentiation of limb primordia and other parts of the amphibian larva by a method having many additional advantages, that of grafting. The part studied was

grafted to another larva of approximately the same age. In such experiments, the grafted parts continue to develop while the graft is nourished and relieved of its excretions by the host. The differentiation of the organs of the host, which are developing simultaneously with those of the transplanted part, must, however, affect the differentiation of the transplant to a greater or lesser degree.

The ideal conditions for a transplantation in which it is desired to determine the independent power of growth and differentiation of the part, would be in a location where there are no nerves from the host, where there is no differentiation of host tissue to influence the grafted part, and where there is a sufficient supply of blood vessels to insure nutrition and the ability to excrete waste products on the part of the transplant. This must be in a rapidly growing tissue which is capable of repair, and such as will react to foreign tissue with rapid incorporation and a host of capillaries. The chorio-allantoic membrane of the chick furnishes such conditions as has been pointed out by Murphy (1913), Danchakoff (1916), Kiyono (1917), Minoura (1921), and Atterbury (1923).

It is my intention to attack the problems of independent growth and differentiation by the isolation and transplantation of the various primordia of the chick embryo to the chorio-allantoic membrane. A general survey of the field has already been made with a variety of tissues and has yielded promising results. For instance, when a cross section of the body of the thirty-six or forty-eight hour chick is transplanted, the grafts obtained show that cartilage, bone, muscle, nervous tissue, and mesonephros grow well. In one case of transplantation of the primitive streak, nephrogenous tissue is found to be present. In many cases of such grafts of cross sections of the body, small buds of the feather germs form and grow well; they receive a rich vascular supply. The present paper is confined to the organs of special sense, *i.e.*, the eye, nasal region, otic region, and one example of brain tissue, the mesencephalon. The mesencephalon was selected because of its close association with the eye, and because of its high degree of differentiation in the birds. Other parts will be considered in later papers.

The progamme was suggested by Prof. F. R. Lillie of this laboratory to whom I wish to express my appreciation for many helpful suggestions and criticisms during the progress of the work.

## II. Methods.

In the experiments recorded in this contribution, I have used chicks of twenty-four, thirty-six, and forty-eight hours of incubation as the source of material. The methods of grafting employed are similar to those used by Murphy and Danchakoff with certain modifications developed at this laboratory. An egg of nine to ten days of incubation is candled and the juncture of two of the larger blood vessels is marked. The shell is then sterilized and a small window is cut through it at this point with a hacksaw blade. The egg is then returned to the incubator while the embryo which is to furnish the material for the transplantation is put in physiological salt solution on a warm stage under a binocular dissection microscope. For illumination, the light of a 150-watt, nitrogen-filled tungsten lamp is concentrated on the embryo by a bull's-eye condenser. All dishes are sterilized in the autoclave and all instruments are boiled in water. Dissecting needles which have been ground into fine knives are used in the operations. With these, the primordium is isolated and is then placed on the chorio-allantoic membrane as near as possible to the juncture of the two vessels noted above. The shell membrane which is torn slightly to admit the tissue is replaced, the shell is returned and the cut edges are sealed with paraffine. The egg is then returned to the incubator in such a position that the window is down, so that the weight of the egg insures the contact of the membrane and the implanted tissue. After twelve hours the egg is rotated, and at regular intervals thereafter rolled as any developing egg. Many of the transplants are sure to have more or less mechanical injury, and the time required for their incorporation within the membrane of the host and subsequent infiltration with blood vessels must vary. These are the most important variables with which the method must cope. After six or seven days the egg is opened and the development of the grafted tissue is studied macro- and microscopically.

## III. Experiments.

### *A. The Eye.*

A series of grafts of the optic primordium of the chick have been made by this method. The material was taken from chick embryos of twenty-four, thirty-six, forty-eight, and in a few cases sixty hours of incubation. In some cases the entire primordium was excised, in others only a part of it was used. There are various difficulties to be encountered in such an operation. The mesenchyme, at these early stages, is quite sticky, and considerable care must be exercised to remove the greater part of it from the vesicle. Particular care is needed if it is desired that no part of the brain be included in the transplants. At the twenty-four hour stage, the neural folds are open in the optic region and only a slight swelling indicates the position of the optic foveola. The anterior end of the head was removed from such embryos; the region from which the optic vesicles develop was then bisected, and the primordium of each side was transplanted separately. The lens ectoderm is not yet definable so that transplantation of this region with that of the future optic vesicle is a matter of chance. At the thirty-six hour stage, the technic is much more simple. Here the embryo has from twelve to sixteen somites. Some of the embryos of this age were more advanced, but the majority fall within these limits. The primordium of the eye is then in the vesicle stage and is easily removed. Great care must be taken that the tissue is not harmed in the transplantation.

Another set of experiments was made on the eye at the thirty-six hour stage. The main part of the experiment was the same, but the lens ectoderm was dissected away. This is very difficult to do, for there is a great tendency for the wall of the optic vesicle to tear. In most of the cases which have been operated in this way, no lens ectoderm was left on the vesicle, but one case which later developed small portions of lens tissue is in doubt. The mechanical injury in an operation, must, of necessity, be great, and this is probably the cause of the greater degree of disorganization found in these grafts. Grafts of the isolated lens ectoderm were also made but no growth was found in any case, possibly owing to the very small size of the piece of the embryo transplanted.

With the experiments made from the eye primordium of the forty-eight-hour embryo, greater difficulties with the elimination of the surrounding tissue began to appear. It is practically impossible to isolate absolutely the optic cup of this stage from the surrounding mesenchyme without injuring the cup in some way. The optic cup can be dissected out with little difficulty, but in every case there may be seen a fur-like border of the mesenchymal cells attached to its surface. After a few attempts to remove this, it was considered to be useless, for the manipulation injured the cup in every case. On this account, when a transplantation of the optic cup is spoken of, it means that the optic cup together with the immediate mesenchyme was taken. Attempts were made here, as in the experiments with the thirty-six hour embryos, to remove the lens primordium from the optic cup. It was soon found that, though this is possible, there is a great deal of mechanical injury involved. Such experiments were therefore omitted. Since the sixty-hour "eye" is in much the same condition, the same difficulties were experienced with it, but it was not considered necessary to perform many experiments with the material from this age, for even at thirty-six hours of incubation, the cells of the optic primordium possess complete power of independent self-differentiation.

In the grafts obtained from the foveola of the twenty-four hour embryo, there is much variability in growth and differentiation. This seems to be due to the fact that there is a certain amount of crushing in the removal of the tissue, the disorganization produced by this mechanical factor increasing as the size of the operated part deceases. In one of the grafts there is a primary differentiation into tapetal cells which contain pigment granules, and retina-forming cells. Many of the grafts show nervous tissue but it is not sufficiently differentiated to warrant any positive statement as to its nature. There is much other tissue transplanted with the optic primordium of this age. In one of the grafts well defined ganglion cells are present. In no case, however, does any eye graft of this age show more than the primary differentiation into pigmented tapetal cells and unpigmented retina-forming cells.

The grafts of the optic vesicle plus the lens ectoderm of the

thirty-six hour embryo will be considered next. At this stage the optic primordium possesses great power of independent growth and differentiation. Many grafts were obtained and sectioned, a number of the most successful of which will be discussed here.

FIG. 1. Graft of an eye from a forty-eight hour chick embryo in alcohol before section, 19 *E* 13. (× 10.) For explanation, see text, page 290.

All of the cases which show any growth at all, prove, on microscopical examination, to have a primary differentiation of the tissues into a pigmented tapetal portion, a non-pigmented retinal portion, and a group of lens-forming cells. The results vary all the way from grafts in which there is no great differentiation of the retina and lens, to the more successful ones in which the retina is differentiated and the whole transplant appears approximately normal.

The more successful grafts show a high degree of differentiation though great variation in the extent to which the subordinate parts of the organ have differentiated. It is an interesting fact that the basal layer of the retina often shows many cells in active mitosis, *i.e.*, the graft is still in active growth. In the best graft obtained (Fig. 2), there is a very clearly defined retina with the layers developed sufficiently to warrant the statement that it is as far advanced as the normal; the lens is perfect, showing the anterior epithelial layer and the lens nucleus; the anterior and posterior chambers are present; the tapetum and the cartilage

of the eye are typical of the normal of the same age. The ciliary processes show around the lens. This case, 9 A 1, is therefore selected for detailed description.

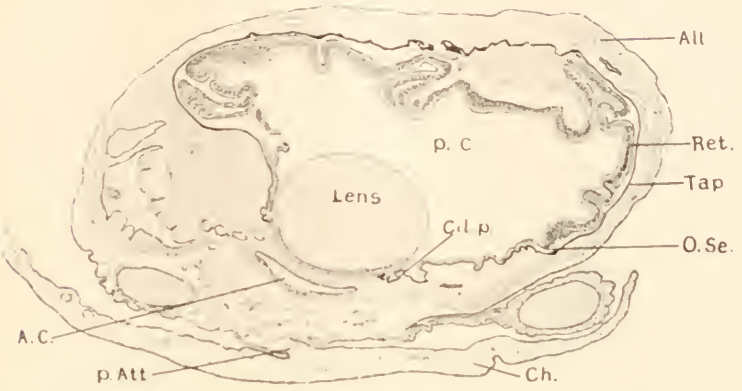


FIG. 2. Cross section of graft of eye grown from thirty-six hour primordium. Total age, nine and one-half days, 9 A 1. (× 20.) *A.C.*, anterior chamber. *All.*, allantoic portion of investing membrane. *Ch.*, chorionic portion of membrane. *Cil p.*, ciliary process. *O. Se.*, ora serrata. *P. Att.*, point of attachment of the graft to the main portion of the membrane. *P.C.*, posterior chamber. *Ret.*, retina. *Tap.*, tapetum.

On March 24, 1922, the optic vesicle of a thirty-six hour chick was removed as free from adjacent parts as possible and was placed near the juncture of two of the largest blood vessels on the chorio-allantoic membrane of a chick of nine and one-half days' incubation. The egg was then returned to the incubator where development proceeded until April fourth when the egg was opened and the graft, which was large, removed. In my notes, taken at the time, I notice the remark that the graft was heavily pigmented.

Upon microscopical examination of the sections, the degree of differentiation of the graft proved to be quite remarkable. In only a few places is mitosis in the basal retinal layer common, *i.e.*, at places where there are large folds protruding into the posterior chamber. By far the greater part of the tissue has ceased proliferating and is differentiating. It may be well to consider this differentiation under four headings; the chambers, the tapetum and retina, the lenticular region, and the surrounding tissue.

The chambers in this case are well developed. A definite anterior and posterior chamber are present separated by the lens, the lenticular zone, and a thin layer of mesenchymatous tissue which is covered with an endothelium. The entire anterior chamber is lined by this tissue. The posterior chamber is large and is lined throughout by the retina save at the anterior border, where the lens and ciliary processes occur, as mentioned above. It is not regular, however, for at intervals, as can be seen (Fig. 2), the retina has proliferated to such an extent that there are folds entering the cavity. In no place do these obliterate the chamber as in some of the grafts.

The retina and the tapetum are well formed, though where convolutions of the retina occur, the tapetum does not always follow. On close examination, the tapetum appears to be formed of three or four layers of heavily pigmented cells arranged very closely. All of the layers of the retina are present (Fig. 3) but the layer of

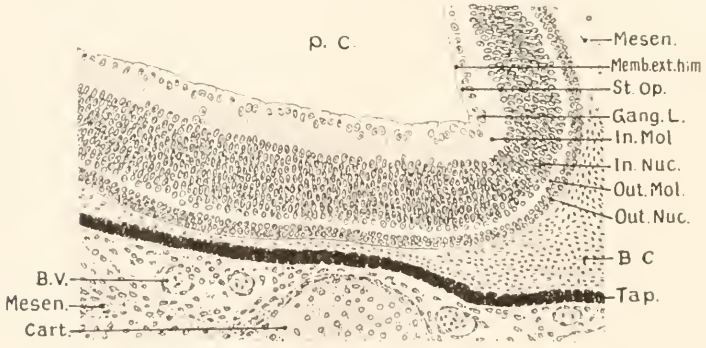


FIG. 3. Cross section of retina from same graft as Fig. 2. (× 180.) *B.C.*, blood corpuscles. *B.V.*, blood vessels. *Cart.*, cartilage. *Gang.L.*, ganglionic layer. *In.Mol.*, inner molecular layer. *In.Nuc.*, inner nuclear layer. *Memb. Ext.Lim.*, external membrana limitans. *Mesen.*, mesenchyme. *Out.Mol.*, outer molecular layer. *Out.Nuc.*, outer nuclear layer. *St.Op.*, stratum opticum. Other abbreviations as in Fig. 2.

rods and cones shows but little differentiation, a condition corresponding to that found in normal eyes of chicks of the same age. Bordering the cavity is the very thin membrana limitans interna. This separates the cavity of the eye from the stratum opticum which lies just beneath it. This is clear and easily distinguished from the sparsely nucleated outer ganglionic layer. Between this

and the inner nuclear layer is an inner molecular layer, very granular in appearance and containing only a very few scattered nuclei which have failed to associate as yet with either of the adjoining layers. The inner nuclear layer is nearly three times as wide as the molecular layer and is very heavily nucleated. Separating this from the outer nuclear layer is the outer molecular layer, also free of nuclei. As mentioned above, the rods and cones have not differentiated at this stage. At places, especially in the region of the retinal folds, there are connective tissue cells which fill in the spaces between these folds and the tapetum.

The differentiation occurring in the lenticular region is quite striking (Fig. 4). The tapetum continues anteriorly to a point

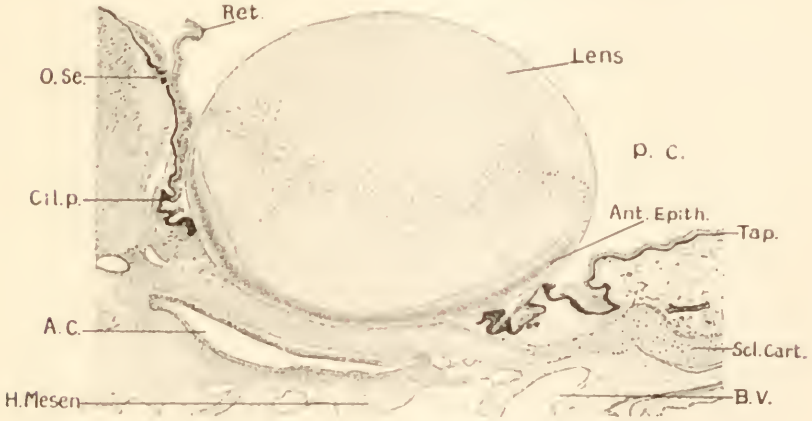


FIG. 4. Lens and lenticular zone of same graft as Figs. 2 and 3. (× 50.) *Ant.Epith.*, anterior epithelium of lens. *H.Mesen.*, host mesenchyme. Other abbreviations as in Figs. 2 and 3.

just mediad to the margin of the lens where it doubles on itself in a pigmentless layer of cells. The ora serrata, ciliary processes, and the iris are present. The lens itself is supported by the suspensory ligament.

In its differentiation, the lens is quite typical (Fig. 4). It is oval in shape, being composed of a wide nucleus of fibers with their medially located nuclei, and an anterior epithelial layer. Both are identical with the same parts of the normal of the same age. The lens surface is toward the ectodermal portion of the chorio-allantoic membrane. The incorporation of the graft

within this membrane was so complete that no injury was done to these parts when the membrane containing the graft was removed from the shell of the host.

Very little tissue save those already mentioned occurs in this graft. The sclerotic coat of the eye is present throughout. To one side there is a small mass of brain tissue containing typical nerve cells and processes. This is heavily infiltrated with blood vessels and not easily identified. Adjacent to this region, the tapetum and retina are very much convoluted and disorganized, and a heavy infiltration of blood corpuscles among the folds makes the identification of definite structures impossible. The optic nerve and pecten which would be located in this area under normal conditions cannot be found. The disorganization and the infiltration of the blood cells may mask their position as degenerative changes are apparent. The entire graft is surrounded by the mesenchymatous tissue of the host.

In not all of the grafts is brain tissue present. A graft of the optic region of a thirty-five hour chick, case 14 *A* 1, when sectioned shows a very evenly shaped posterior chamber lined by well differentiated retina. This eye shows the same differentiation of retina and tapetum as graft 9 *A* 1 above, though there is a total absence of brain and cartilage tissue. The various layers of the retina are well defined. The cut edges of the excised optic vesicle have drawn together and the point at which they have fused is marked by a thickening and by slight convolutions of both tapetum and retina. No lens tissue is present in the sections, probably due to the failure of its primordium to be incorporated. The grafted tissue is surrounded by an extremely thin layer of host mesenchyme. In no place is there any sign of brain tissue, optic nerve, or pecten, though the point at which they would normally occur is definable.

Graft 19 *E* 13 from a forty-eight hour chick embryo, is somewhat similar to 9 *A* 1 above. This case, after seven days of growth, a total of nine days, showed a graft 4 mm. x 4 mm. in size. A drawing of this graft as it appeared in alcohol before sectioning is shown in Fig. 1. The heavy pigmentation of the greater portion of the surface will be noted, together with the absence of pigment from a portion of the upper face. It was

thought on macroscopic examination, that this would prove to be the lens, but on section, a very different picture presented itself. The unpigmented portion is the point at which the optic nerve leaves the retinal area and runs out into the mesenchyme (Fig. 5).

FIG. 5. Cross section of origin of optic nerve in graft, 19 *E* 13. Same as Fig. 1. (× 140.) *Br.*, brain. *Cart.*, cartilage. *Opt.N.*, optic nerve. *Pect.*, pecten. Other abbreviations as above.

The pecten is present though irregular in shape, due no doubt to the mechanical stress in the growth after transplantation. From this point the optic nerve grows out between the tapetum and the ectodermal wall of the chorionic membrane for quite a distance. The other white area in the graft is brain tissue which was transplanted with the optic cup. Only in the region of the nerve and nervous tissue is there any mesenchyme or cartilage in abundance. In the rest of the graft, the tapetum comes up close to the border of the chorionic or the allantoic mesenchyme of the membrane. The layers of the retina are all well differentiated. The ora serrata appears near the margin of a much distorted lens, ending on its lenticular border in a much convoluted and disorganized ciliary process. The misshapen appearance of the lens is apparently due to the fact that it lies next to the shell membrane and

is not completely incorporated by the chorionic overgrowth. At both ends of the sectioned material, where the stratum opticum is cut nearly transversely, the nerve fibers of this layer show very well. The whole graft compares very favorably with the normal eye of the same age.

In the experiments performed on the forty-eight hour chick, as in those cited for the thirty-six hour material, the degree of regulation seems to be dependent on the amount of injury to the engrafted tissue, both during manipulation and during the growth period which follows. My data show many cases where a much convoluted retina and tapetum are present with no posterior chamber showing. All degrees of lens malformation may occur. The retinal cells are always definable as such, though the degree of differentiation of the retina as expressed by zone formation varies considerably.

Five successful grafts were obtained from thirty-six hour primordia from which the lens ectoderm had been dissected away. The data on these cases are to be found in table I. In only one of

TABLE I.

GROWTH OF THIRTY-SIX HOUR OPTIC VESICLES WITH THE LENS ECTODERM REMOVED.

| Case. | Control Age. | Remarks. |
|---|---|---|
| 15 *A* 1 | 8½ days | Posterior chamber, retina and tapetum present. No lens tissue. |
| 15 *B* 1 | 8½ days | Nervous tissue, cartilage, much convoluted retina, tapetum, small amount of lentoid tissue. |
| 15 *C* 1 | 8½ days | Very little area of much convoluted retina and tapetum only. |
| 16 *B* 1 | 8 days | Nervous tissue, retina and tapetum. No lens tissue. |
| 16 *B* 2 | 8 days | Cartilage, retina, much convoluted tapetum. No lens tissue. |

these grafts are any lens cells present. It is more reasonable to suppose that these are the result of the proliferation of some cells of the lens ectoderm which were not removed in the operation, than that they arose by a redifferentiation of the cells of the retina or iris (see Fischel for amphibian larvæ, 1900) or by a stimulation of the chorionic ectoderm by the optic cup (see Lewis 1907 and Spemann 1912, and other papers on the amphibian eye).

Spemann (1912), Lewis (1907 *a*, *b*, *c*,), and other workers have

found that the optic cup of the amphibian embryo has great power of self-differentiation when transplanted to other parts of the same or another embryo, while the lens ectoderm is dependent on contact with the optic cup for its development. The specificity of the ectoderm which forms the lens varies with the species employed. Lewis (1907 *c*) notes that the various parts of the cup develop independently "in marked contrast" to the lens which will not form unless the optic cup is present. If brain also is present, there may or may not be an optic nerve developed. The tissues seem to continue their own specific type of differentiation independent of one another.

The fact that in case 14 *A* 1 where no brain tissue is present, the optic nerve is also lacking, while in case 19 *E* 13 both are present, seems, therefore, to deserve more than passing notice. In the first mentioned case, the point of fusion of the cut edges of the primordium is definable. There is no indication of the optic nerve anywhere in this region. In case 9 *A* 1, while there is a small amount of brain tissue present, the position of the optic nerve cannot be found. It may be obliterated by the great number of degenerate convolutions in the retina and tapetum adjoining this region. In case 19 *E* 13, however, both optic nerve and pecten are very definitely located. The position of the optic nerve is the same as that of the optic stalk which connects the optic cup with the brain in the early stages of development. When this is removed, the optic nerve fails to develop. The fibers of the cells, instead of finding exit by this path, are retained in the stratum opticum. It would seem that these structures, as well as the parts of the retina and lenticular region arise from very definite locations in the primordium.

The experiments show very definitely that the various parts of the eye primordium have the power of independent self-differentiation to quite a remarkable extent. This conclusion is strengthened by the findings of Lewis, Spemann and others for the amphibian eye. The regulation of the parts to form a more or less perfect structure seems, on the other hand, to be dependent on various mechanical factors involved in the experiment.

### *B. The Nasal Region.*

The material for the grafts of the nasal region was taken from chicks of thirty-six, forty-eight, and sixty hours of incubation. As in the case of the eye tissues, only a few grafts were made of the sixty hour material. Inasmuch as it was desired to note the growth and differentiation of the nasal epithelium especially, no twenty-four hour material was grafted, for at this age, the medullary folds only are localized.

When grafts were made of thirty-six hour chick primordia, the anterior end of the head as far back as the optic vesicles was transplanted. If the material was taken from an embryo of forty-eight hours incubation, the tip of the head was cut away so that both nasal pits were transplanted in one piece.

A brief account of the normal development of the olfactory organ based on Disse's account (1897) will form a useful introduction to the study of the grafts of this region to be described. After three days of incubation, the sensory epithelium of the chick nose is represented by two thickenings of the epithelium in the nasal grooves. As the embryo approaches the fourth day, the epithelium becomes differentiated into columnar epithelial cells and round cells, more transparent in sections stained with carmine. These cells are isolated in groups which are more or less distinct from each other. It is from these that the cells migrate out, forming the olfactory nerve and the ganglion-like group. At first a chain of cells appears to lead from the nasal epithelium to the brain. As late as the sixth day, this nerve primordium contains cell bodies but they are most numerous at the extremities. In the eight day chick, the stage which serves as the control for the grafts to be described below, Disse finds the nerve formed and two types of cells appearing in it at rare intervals. There is a pear-shaped unipolar cell, and a bipolar cell that is spindle-shaped. The greater part of the nerve is composed of nerve fibers. The cells are most numerous at the ends of this fiber bundle. At the distal end, near the nasal epithelium, the grouping of these cells and their shape led Beard (1885) to describe a ganglion-like mass which Disse describes merely as a group of cell bodies. Disse (confirming His, 1889) concludes that the nervus olfactorius arises from certain cells of the nasal epithelium and adds that it lacks a ganglionic portion.

The cartilages of the nasal capsule of the chick have been described by Cohn (1903). The prenasal cartilages and the nasal septum are present on the eighth day. The primordia of the three turbinals are formed. These are the only cartilages which arise from the mesenchyme transplanted in the grafts.

Graft 41 *B* 1 is a growth of the nasal region of a chick of thirty-six hours of incubation. The graft was removed after six days of growth on the chorio-allantoic membrane of the host, a total of seven and one-half days, and was 3 mm. x 3 mm. in size. On section, the various parts of the nasal region are found to be present. They compare favorably in their development with the normal region in the embryo of eight days of incubation.

The grafted tissues with the exception of a portion of the ectoderm are well surrounded by the mesenchymatous tissue of the host membrane. In it, four gross areas are definable, the nasal sacs, the nasal cartilages, the forebrain, and the epidermis of the head. With the exception of the epidermis, these have maintained approximately their normal relationships. The sections cut the region transversely so that at one end the nasal sacs appear, separated by the cartilage of the nasal septum. As the sections are traced posteriorly, the sacs become surrounded by a cartilaginous sheath, the prenasal cartilages. As the nasal sacs disappear posteriorly, the forebrain appears. It is heavily infiltrated with blood corpuscles which do not seem to be located in any organized vessels. Between these structures themselves, and separating them from the chorionic and allantoic borders of the host membrane is a good growth of mesenchyme. A more detailed description of these parts follows.

The nasal sacs are two in number and distinct (Fig. 6). They end blindly at both ends and are completely surrounded by mesenchyme, save at one place where the left sac retains its connection anteriorly with the epidermis of the head. Posteriorly they widen out forming cavities of some size. The epithelium which lines the cavities is columnar in type. Mitosis is fairly common in the entire region but is more commonly found near the cephalic border where the epithelium is noticeably thickened. For the most part this layer is sharply defined from the surrounding mesenchyme, so that it appears as if a membrane were present

surrounding it. In the thickened areas, however, the boundary becomes indistinct, and in many places, cells may be seen which appear to be migrating from the epithelial layer into the mesenchyme. These areas represent the sensory epithelium and from them the olfactory nerve arises. A short distance from the nasal

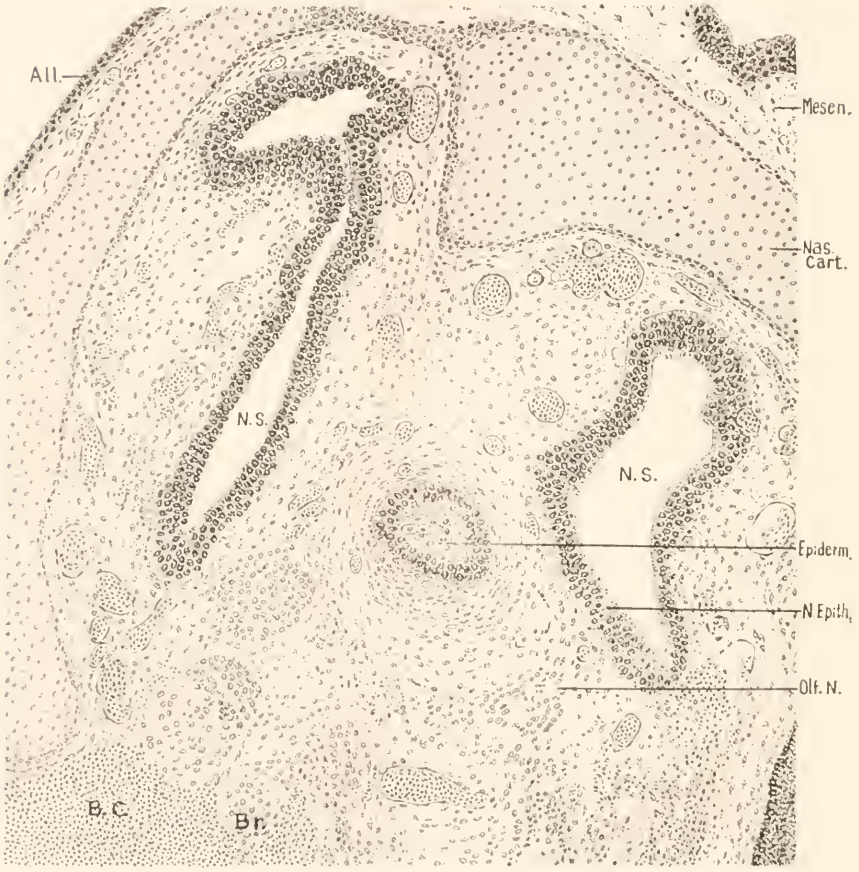


FIG. 6. Cross section of graft of nasal region of chick embryo of thirty-six hours of incubation, 41 *B* 1. (× 140.) *All.*, allantoic border of membrane surrounding graft. *B.C.*, blood corpuscles. *Br.*, brain. *Epderm.*, epidermis. *Mesen.*, host mesenchyme. *N.Epith.*, nasal epithelium. *N.S.*, nasal sacs. *Nas.Cart.*, prenasal cartilage. *Olf.N.*, olfactory nerve.

epithelium may be seen a small group of ganglion-like cells (Marshall, 1879) and toward these the nerve cells appear to be migrating. From the ganglionic group, nerve fiber bundles con-

taining a few cell bodies which correspond to the pear-shaped and spindle-shaped nerve cells of Disse, run to the forebrain where their course is lost in the disorganization present there. The large round primordial nerve cells of the nasal epithelium occur in the fiber bundles at rare intervals. The development of the nerve corresponds to that of the embryo of seven days of incubation as described by Disse.

The forebrain of the graft has no limiting membrane. The character of the cells is undoubtably that of brain cells but they are unorganized. This appears to be due to the fact that this tissue is so extensively infiltrated with blood vessels. In places there are what seem to be large sinuses filled with blood cells. Numerous small nerve-like masses are found in this region which are formed by union of the groups of the nerve processes. They end bluntly and blindly after penetrating the mesenchyme for short distances, resembling similar structures found in grafts of the mesencephalon which will be discussed below.

The cartilage which surrounds the nasal sacs has been mentioned above. This cartilage is in a single large piece but at intervals in the sections, there are breaks in its continuity. The nasal septum is present as a T-shaped structure, the top of the T forming a part of the floor of the region. Forming a roof over the nasal sacs is a crescent-shaped prenasal cartilage. This is broken at a single place on one side to permit the exit of a blood vessel. The cartilage does not persist through the entire graft but disappears shortly beyond the posterior end of the nasal sacs.

The epidermis of the head is present in the anterior end of the graft as an extensive, more or less cornified mass of tissue imbedded in mesenchyme. As it is traced posteriorly it approaches and finally, at the posterior level of the olfactory sacs, reaches the chorionic surface of the membrane where it is unincorporated by the host. A single tube-like growth of this epidermis has come to lie between the tip of the left olfactory sac and the forebrain.

As far as I have been able to discover, the only data on the differentiation of the nasal epithelium when isolated either completely or together with the immediate region, are furnished by Lewis (1907 *d*). This author records two experiments which show in the first place that the epithelium develops independently

of the forebrain, and secondly, that the nasal pit is capable of independent differentiation. Both sets of experiments were done with the larva of *Amblystoma*. In the first type of experiment, though the forebrain primordium was removed, the nasal epithelium sent out nerve processes into the adjacent mesenchyme, developing normally. The second type of experiment involve the transplantation of the excised nasal pits to a position beneath the ectoderm dorsal to the eye. Lewis (page 464) states that ". . . differentiation of the organ continued and nerve fibers were sent off into the region between it and the ectoderm, . . . but extend only a short distance."

In the grafts obtained from the transplantation of the nasal region of the chick embryo, the different parts of the organ show a high degree of specificity in the course of their differentiation. In comparing them with the control and with the description of the process of nerve formation by Disse, it was found that they show a condition comparable to growth in a normal embryo of the same age. The cartilage which is present, is, without a doubt, that of the transplanted part, and not of the host. The nasal sacs are present in nearly their normal relationships to the rest of the parts and have a differentiation closely resembling that of the same parts in the normal organ. The nasal epithelium gives rise to the nerve which is also present. The nerve cells of the forebrain differentiate nerve processes, the abnormal appearance in the sections being due to excessive vascularization and the absence of the limiting membrane, both of which are mechanical factors controlling the limits of differentiation to a great degree.

### *C. The Otic Region.*

Grafts of the otic region and the otic vesicle are much more difficult to make than those of the nasal region. The auditory pit appears about the thirty-six hour stage, and the vesicle is closed about the forty-eight hour stage; the material grafted was taken from embryos of the last mentioned age in most cases. The first experiments were made by shelling out the otocyst and transplanting it, but it appeared very soon that the otocyst alone does not persist well in grafts. The mechanical injury due to the pressure of the egg itself, together with the adhesive quality of

the shell membrane due to its absorptive nature, resulted in no incorporation in the membrane of the host, and consequently no growth. I then tried transplanting the otic vesicle with the immediately adjacent mesenchyme, a slightly larger piece of tissue, and found that in such operations, though incorporation may not be complete and the mechanical injury more or less extensive, nevertheless, growth ensued. The investing tissue transplanted at the forty-eight hour stage includes only mesenchyme and somatic ectoderm. In all the operations, the brain wall was removed from the transplant before it was placed on the membrane of host.

Table II. is a record of the successful growths of the auditory

TABLE II.

DATA ON GRAFTS OF THE OTIC REGION.

| Case. | Donor Age. | Control Age. | Remarks. |
|---|---|---|---|
| 24 *D* 7 | 36 hr., 20 Som. | 9½ days | Cartilage in crescent around epithelium of otic vesicle; epithelium differentiated. Incomplete incorporation. |
| 25 *C* 5 | 48 hr | 9½ days | Cartilage independent of differentiated epithelium. Incomplete incorporation. |
| 32 *A* 5 | 52 hr. | 9¼ days | Differentiated epithelium in two parts, one surrounded by cartilage. Incomplete incorporation. |
| 32 *B* 5 | 52 hr. | 9¼ days | Cartilage near though not surrounding incompletely incorporated, differentiated epithelium. |
| 37 *C* 5 | 48 hr. | 8 days | Cartilage near epithelium. Small graft, epithelium differentiated. |
| 38 *A* 5 | 48 hr. | 8 days | Only small amount of unincorporated epithelium which is differentiated. |
| 40 *B* 6 | 48 hr. | 9 days | Cartilage and epithelium alone present. Epithelium differentiated. |

vesicle. It will be noted that one of these is from a donor of thirty-six hours incubation, but the embryo showed a more advanced stage of development than is usual for chicks of that age. It corresponds more nearly to the forty-four-hour stage and consequently approaches the usual donor age, forty-eight hours.

Two criteria are employed in the identification of the parts of growths obtained in grafts of the otocyst. The morphological arrangement of the various structures aids greatly, but owing to the disorganization present in every case, this is supplemented by

close histological examination. In normal development, the cartilaginous otic capsule surrounds the sacculus, utriculus, and their derivatives, while the ductus and the saccus endolymphaticus are free in the mesenchyme. The histological differentiation of the several parts of the membranous labyrinth in the grafts is essentially the same as that of the control so that identification can be made positively by histological structure; this is supplemented as stated above by the general morphological relationships present.

The differentiation attained by the grafted tissues is of two kinds, cartilaginous and epithelial. No ganglion cells or other nervous tissue of any kind can be found in the grafts. This fact emphasizes the isolation of the primordium before its transplantation, for the ganglion and the otocyst are very closely associated at that early stage. The cartilage partially surrounds the epithelium, the latter forming the lining of cavities of various sizes. The relationships of the various parts of the organ is not normal. I have selected for description two cases which show quite an extensive specific differentiation of the membranous labyrinth.

Graft 32 *A* 5 is a growth obtained from the transplantation of a forty-eight hour primordium which has remained in the host membrane for six days, a total of eight days growth. Completely surrounding a large epithelial sac is a capsule of cartilage which is discontinuous in two places. Through one gap, small blood vessels enter a narrow group of mesenchymal cells which separate the epithelium of this region from the cartilage surrounding it. The other break in the cartilage sheath is in a place where an incompletely incorporated portion of the epithelium makes a connection with the epithelial sac mentioned above. The cartilage, from its general relations, is undoubtably the otic capsule.

The epithelium of the membranous labyrinth shows a high degree of specificity in its mode of differentiation. It may be divided into two main parts, that incompletely incorporated and outside of the cartilaginous capsule, and that within the capsule. The epithelium which is outside of the cartilage is histologically identical with that of the normal saccus endolymphaticus, the part connecting the saccus endolymphaticus with the epithelium of the capsule, the ductus endolymphaticus. These two portions

are surrounded by mesenchyme only, as in the control. The parts within the cartilaginous capsule show a high degree of differentiation also. In the region of the vascular foramen referred to above, the epithelium has the characteristics of the normal utriculus of the eight day chick embryo. This determination is further confirmed by the appearance in the epithelium of three thickened areas which represent the cristæ acusticæ of the ampullæ of the semicircular canals. No ganglion cells are associated with these as in the controls. There are no canals or ampullæ formed though at a point occupied by one of the cristæ (Fig. 7.

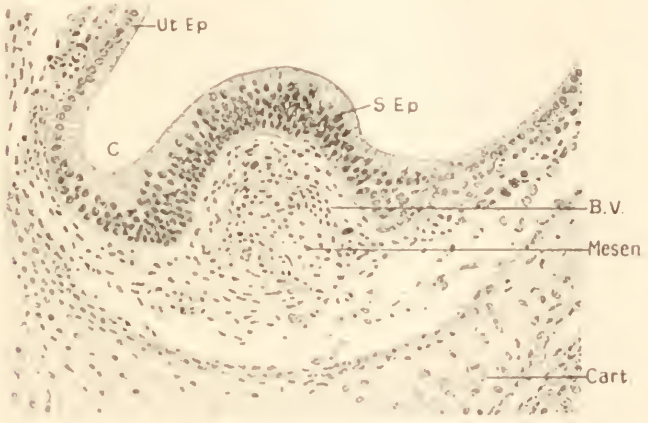


FIG. 7. Section of graft of otic vesicle of forty-eight hour chick in region of one of the cristæ acusticæ. Eight days growth. 32 : 1 5. (× 235.) *B.V.*, blood vessels. *C.*, evagination suggesting beginning of canal formation. *Cart.*, cartilage of otic capsule. *Mesen.*, mesenchyme containing blood vessels. *S.Ep.*, crista acustica. *Ut.Ep.*, utricular epithelium.

Compare Fig. 8) there is a slight evagination which suggests the beginning of such formation. It is very limited in extent, appearing for only a few sections. Through the space formed by the second break in the cartilage, the large sac lined by utricular epithelium is connected to the endolymphatic derivatives mentioned above. A few sections beyond this the wall of the sac shows a differentiation into a stratified sacculus-like and a thinner utricular epithelium. The ductus cochlearis and the lagena which normally arise from the sacculus are absent in the graft. It may very well be that the presence of the cartilage sheath has acted as a mechanical obstruction to their formation.

The same definite differentiations of the epithelium show well in case 40 *B* 6. Here, as in the case cited above, both cartilage and epithelium are present. The cartilage is not so extensive, however, but is located near, though not surrounding, the epithelial tissues. The relations of the parts of the membranous labyrinth are very much disorganized in this graft, the tissue

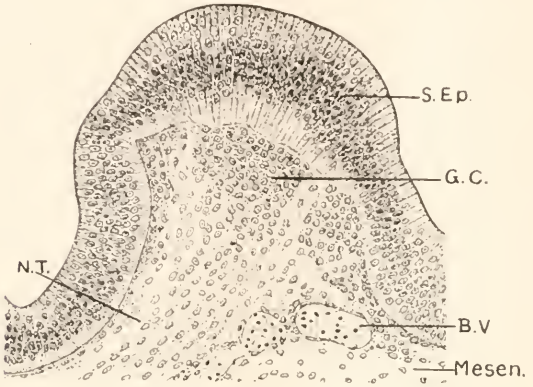


FIG. 8. Section of crista acustica of one of the ampullæ of a chick of eight days incubation. (× 235.) *G.C.*, ganglion cells. *N.T.*, nerve tract running from crista to brain. Other abbreviations as in Fig. 7.

appearing in two places, separated by the host mesenchyme. The character of the cellular differentiation is distinctive. In one of the groups, the differentiation is like that of the ductus and saccus endolymphaticus. These structures are connected, the ductus appearing as a tube leading from the saccus. Near these, a canal-like formation appears for a few sections These parts are not widely separated from the other group of structures which are adjacent to the mass of cartilage. The parts in this group are in the form of distinct vesicles and tubules, resembling histologically, utriculus, sacculus and a small portion of a canal. In the utricular wall are two areas interpreted as cristæ acusticæ, one of which is associated with the canal-like portion already mentioned.

It would seem therefore, from the data given for the above cases, that the differentiation of the constituent parts of the otic vesicle is very specific, and that it takes place independent of the relationships of the various parts, which depend on mechanical factors.

Streeter (1907, 1914), who has experimented extensively on the development of the ear vesicle in the amphibian larva, has noted the ability of the vesicle to develop in a nearly normal manner when transplanted to other portions of the head either of the same or opposite side. In most of the cases, it reorients itself as to dorso-ventrality and retains its definite character as from the right or the left side. In Spemann's experiments (1910), the inverted otocyst did not orient itself in every case, due, in Streeter's opinion, to the fact that in making the large incision for the rotation of the vesicle, the adjacent tissue was much disturbed and also to the factor of mechanical pressure which was applied to insure the closure of the wound. Streeter eliminated these factors by making one slit and stretching this for the operation. Closure and healing thus took place immediately without the application of pressure. Spemann's results do not otherwise seem to be in conflict with those of Streeter. The results of both of these investigators emphasize the high degree of independent self-differentiation obtained by the organ. Streeter notes (1914, page 172), "All our evidence points to a high degree of differentiation of the cells of the vesicle, and it is conspicuously proven by their possession of laterality, . . ."

It will be of interest to note also a case cited by Lewis (1907), in speaking of which he states that an otic capsule of the host, *Amblystoma*, formed around the membranous labyrinth arising from transplanted tissue from *Rana sylvatica*. He adds that the character of the cartilage cells is such as to determine them as of Amblystomal origin. Lewis considers that this shows that in some way, the otic vesicle stimulates the formation of the cartilaginous capsule around it. He says (page 145), "If the cartilaginous capsule about the otic vesicle is dependent upon the influence of the latter for its origin it will probably be found the cartilage of other regions of the embryo is likewise dependent on certain influences in the neighboring structures for its origin from the mesenchyme. . . . A piece of transplanted brain or a transplanted eye, for example, even when close to the cartilage forming about the otic vesicle or central nervous system does not stimulate the formation or growth of cartilage about itself."

In the grafts of the otic vesicle described above, the cartilage

is identified as that of the otic capsule but it is very definitely the result of the differentiation of the cartilage-forming tissue of the graft and not of the host mesenchyme stimulated by the membranous labyrinth. The very fact that it only partially surrounds the parts of the vesicle proves this. It will be recalled that a fringe of mesenchyme was transplanted together with the otic vesicle. This would easily account for any cartilage present in the grafts.

The specificity of the cellular elements of the otic vesicle is very marked (see also Streeter, *loc. cit.*). Utriculus, sacculus, endolymphatic duct and sac, and the canals are recognizable by the character of their histological differentiation. Though the canals have not formed in any of the cases, their general position is indicated by the occurrence of the cristæ acusticæ of their ampullæ. In no case are they accompanied by ganglion cells. Neither is the auditory nerve present in any of the grafts. This indicates a complete isolation of the primordium, for the eighth ganglion is closely associated with the otocyst even at the stage used in the operations. There is therefore no possibility of a stimulation of the epithelium of these sensory areas by nerve cells. These areas are localised in the epithelium itself. By means of various grafting experiments, Harrison (1903) found a similar differentiation of a sensory area, independent of nervous stimulation, to exist in the development of the lateral line organs of *Rana sylvatica* and *Rana palustris*.

In spite of the much altered relationships of the parts of the membranous labyrinth and the surrounding mesenchyme as they develop in the grafts, they differentiate so that they are easily identified in the sections. The amount of adjustment of the relationships of their parts is, on the other hand, very variable, being dependent on the amount of mechanical injury and the position of the grafted tissue at operation, together with the factors of pressure, and stress and strain during the following growth period.

### *D. The Mesencephalon.*

As has been stated above, the selection of the mesencephalon in making grafts of brain tissue was made because of the fact that it is here that the correlation centers of the eye have their seat.

In the adult bird it forms the large corpora bigemina which give off no peripheral nerves.

In the embryo, at about the forty-eight hour stage, the brain wall in this region is sufficiently independent of the ectoderm to enable the isolation of this part of the brain with very little or no mechanical injury. It is comparatively easy to remove all of the metencephalon and the diencephalon from such pieces, so that the transplants contain only mesencephalon. Since such material yields grafts favorable for this study and since the injury at an earlier stage of development is of necessity much greater, the entire series of experiments with this primordium was made with tissue from chicks of forty-eight hours of incubation.

For the sake of comparison, a brief description of the mesencephalon of the eight-day chick will be given here. The whole region is divided into two portions, a basal and a tectal. These surround three connected cavities the larger ones being the mesocœles, and the smaller one, the aqueduct of Sylvius, which is within the basal portion of the region. The differentiation of the wall of the corpora bigemina, the tectum lobi optici, is very definite. If we examine a cross section of part of this region, we find that there are four cortical layers present (Fig. 9). These will be described in the order in which they are found on examination of such a section, from the ependyma to the periphery. Next the mesocœle is an ependyma which is densely nucleated and fairly wide. From it, cell bodies appear to be migrating toward the periphery. The next layer is an inner fiber zone which stands in great contrast to the nucleated zone just mentioned. It is rather wide and contains cords composed of the migrating cell bodies and their processes. The migration of these cell bodies ends in a nuclear layer which lies just peripherad to the inner fiber layer. The processes of these cells extend still further out, forming an outer fiber layer which is much narrower than the inner fiber layer and contains no nuclear strands. Bounding the outer fiber layer is a membrane, the external limiting membrane, on the outer side of which is a thin layer of mesenchyme containing blood vessels.

The outer fiber layer contains processes of the cell bodies of the outer nuclear zone. These are shown very clearly in tangential

sections of the lobe, in which fiber tracts appear, running for some distance across the field. Longer fiber tracts appear in the inner fiber layer. These run into the base of the lobes, through which all of the innervation of this region finds its exit. The basal por-

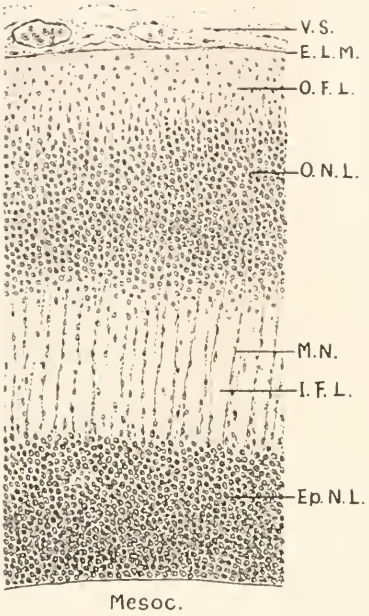


FIG. 9. Cross section of a portion of the tectal part of the mesencephalon of a chick of eight days incubation. (× 180.) *E.L.M.*, external limiting membrane. *Ep.N.L.*, ependymal nuclear layer. *I.F.L.*, inner fiber layer. *Mesoc.*, mesocœle. *M.N.*, nuclear strands. *O.F.L.*, outer fiber layer. *O.N.L.*, outer nuclear layer. *V.S.*, vascular sheath in the mesenchyme.

tion also contains fibers of the ascending and descending nerve tracts. No peripheral nerves are present. The vascularization of the region is accomplished by small vessels which enter the nervous tissue through the external limiting membrane.

Bearing this general condition in mind, an examination of the sections of graft 38 *A* 4 shows, with a few exceptions, a general conformability of its development to that of the normal. The two greater cavities of the control are represented by one large mesocoele. This is bounded by the basal and tectal portions of the grafted tissues. The basal portion gives a rather compact appearance with many fiber tracts. In the cortical region the

differentiation is very striking. The two nuclear layers are present and separated by the inner fiber layer which contains the nuclei in definite strands. In tangential sections this layer also shows many fiber tracts. In this case, this fiber layer also shows a very heavy infiltration of blood vessels, a condition not present in the normal. Such a condition seems to be correlated with

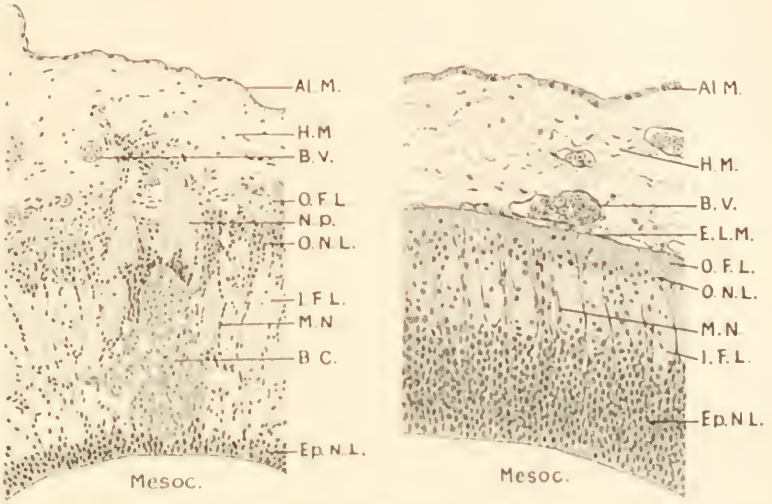


FIG. 10. Section of a portion of a graft of the mesencephalon of a forty-eight hour chick; external limiting membrane not present. 38 *A* 4. (× 180.) *Al.M.*, allantoic border of membrane. *B.C.*, blood corpuscles. *B.V.*, blood vessel. *H.Mesen.*, host mesenchyme. *N.P.*, nerve-like process. Other abbreviations as in Fig. 9.

FIG. 11. Cross section of a portion of a graft of the mesencephalon of a forty-eight hour chick; external limiting membrane present. 18 *B* 5. (× 180.) Abbreviations as in Figs. 9 and 10.

certain other differences in the development of the parts of the primordium, the most evident of which is in the outer fiber layer. In this graft (Fig. 10), the outer fiber layer is not bounded by an external limiting membrane and as a result the fibers penetrate the adjacent mesenchyme. These fibers have grown out into the mesenchyme, or the mesenchymatous tissue of the host has advanced into the nervous tissue of the graft; the width of the zone in which fibers alone are present is very variable. The outer layer may even be lacking in many places, the outer nuclear layer being in immediate contact with the loose mesenchyme

cells of the host, between which the nerve processes make their way. At numerous points along the periphery these processes are united into nerve-like masses which end bluntly at varying distances from their point of origin. Some of the cell bodies of the outer nuclear zone appear to have migrated through the immediately surrounding loose mesenchyme to a more definitely organized envelope of mesenchymal tissue which surrounds the graft and in which run the blood vessels of the host. The structure of this sheath resembles very closely that of the corresponding layer in the normal embryo. It is impossible to say whether it arose from the mesenchyme of the host or of the graft. The blood vessels are those of the host and they show a most extensive development. In places, large spaces filled with blood are present between this layer and the developing mesencephalon. Apparently because of the fact that the external limiting membrane is not present in such places, the vascular infiltration of the fiber layers as well as the nuclear layers is enormous. Even in the mesocœle there are large groups of blood cells though no organized vascular walls appear around them. In the nuclear layers there is a crowding of the developing nerve cells due to the presence of small vessels. In spite of this fact, the layers are distinct. The individual cells of the areas seem to be differentiating in a normal manner.

The significance of the absence of the external limiting membrane becomes evident upon examination of another graft, 18 *B* 5 (Fig. 11), in which this membrane is present around a majority of the cortical tissue. Where it is present, the differentiation of the layers of the cortex corresponds exactly with that of the control. In the peripheral fiber zone, the fibers form a clear band and do not extend into the mesenchyme. As a result, none of the nerve-like processes are found. The vascularization of such regions is much less extensive than that of regions where the membrane is absent. As a result of this, practically no distortion of the nuclear and fiber layers occurs. These are normally proportioned and contain cells which are in the process of normal differentiation.

In other portions of the same graft there are areas lacking the external limiting membrane. As we should expect, the outer

fiber layer is reduced or absent; processes of the nerve cells penetrate the mesenchyme, forming nerve-like structures; and the layers of the cortex are very heavily vascularized.

This graft shows a single large mesocœle as does the other case described. At one end of the sections of both grafts, where the roof of the mesencephalon is cut tangentially, extensive fiber tracts are visible in the inner fiber layer. The mesocœle in both cases is completely enclosed by nervous tissue, the brain having fused at both ends. This was aided in all probability by the apposition of the cut edges at section. The gross morphology of the organ arising from such a graft seems therefore to be affected to quite a considerable extent by the environment though the differentiation of its cellular constituents is specific.

The growth and differentiation of the mesencephalon in grafts made upon the chorio-allantoic membrane of the chick embryo brings out a number of very interesting facts. The basal and the tectal portions retain their individuality though the two cavities are blended into one large mesocœle. The basal portion develops more or less irregularly, dependent apparently on the mechanical factors involved and the absence of ascending and descending tracts when isolated. The tectal portion, however, shows two types of reaction, both of which have already been described. The interesting fact about the development of this part is found in the form taken by the outer fiber layer, dependent on the presence or absence of the external limiting membrane. This also affects the other layers inasmuch as this membrane appears to regulate the penetration of blood vessels from the host (cf. supra). The growth and the differentiation of the various layers is very specific in both cases. The nuclear cords and the width of the layers, with the exception of the outer fiber layer, as has been mentioned above, are approximately normal.

It was mentioned above that in some of the grafts of the mesencephalon and the nasal region, there are small peripheral nerve-like bundles present which do not correspond to any such formation in the normal. These outgrowths of nerve are the result of the differentiation of the individual fibers of the nerve cells, but, owing to the fact that the membrane which furnishes a mechanical obstruction to indefinite outward growth of

these is absent, these fibers have continued to grow into the mesenchyme, forming, in places, nerve-like bundles. Lewis (1907 *d*) describes a similar outgrowth of nerves from the brain tissue of *Rana sylvatica* from which he had removed the optic primordium. He states (page 464): "These nerves extend from a region of the brain which, under normal conditions, never gives rise to peripheral nerves, or at least to nerves that leave the brain in the region to run into the mesenchyme. . . . The injury in the brain in these experiments was done long before the nerves normally appear." The data presented here seem to indicate that the regulation of the growth of the elements is dependent to some degree on the structural environment, and that the disorganization produced by the injury during operation is sufficient to modify the gross structure obtained in development.

## IV. Discussion

The independent power of growth and differentiation of other embryonic parts has been studied to a limited extent by this same method. Danchakoff (1921) has investigated the growth and the differentiation of the blastoderm of the "primitive streak stage" of the chick embryo after transplantation to the "allantoic" membrane. Blastoderms of "ten and more somites" from which the posterior portion to the eighth somite had been removed, were also grafted. In the blastoderms transferred to the allantois at an early primitive streak stage, no differentiation of organs appeared; the implanted parts remain as "islands of undifferentiated tissue." Growths from blastoderms of a late primitive streak stage showing a slight indication of the head process, show groups of cells which "grow well and reach a high degree of differentiation." Eye, nervous tissue, and notochord are present, and in the cartilage which forms around the cord, different parts of normal vertebræ are recognizable. Danchakoff concludes from her results, "the earlier a whole blastoderm is transferred to the allantois, the less growth and differentiation is obtained." While the principles involved in the growth and development of such transplantations are probably the same as those applying to the independent differentiation of the isolated organ primordium, the situation in grafts of such large portions

of the embryo is very complex and for this reason an analysis would be difficult.

More recently Atterbury (1923) has transplanted the metanephric "anlage" of the chick in the same way. The material used in the experiments was taken from seven day chicks and transplanted to the membranes of chicks of the same age. The primordium of the kidney consists of an ureteric channel with many lateral tubules and metanephrogenous tissue at the time transplanted. The differentiation obtained from such grafts after various periods of growth was similar to that of the control metanephros. The isolated metanephric primordium of the chick embryo has thus the power of independent self-differentiation as have the organs studied in this report.

A general survey of the power of growth and differentiation of isolated parts of the embryo was made as a preliminary to the present study. The grafts obtained from implants of cross sections of the somitic region and the primitive streak confirm the results that Danchakoff has obtained for the "blastoderm in toto" of the younger embryo. Cartilage, muscle, nervous tissue, nephrogenous tissue, and epidermis differentiate well. Some of the muscles of the somitic region show very definite striations. The cartilage suggests the form it would have assumed in the normal embryo and the spinal nerves have a serial arrangement indicating the segmentation in the cord.

If the data dealing with more or less extensive organ complexes are compared with the results obtained in the investigation of the isolated primordium of the single organ, it appears that in both cases, the differentiation of the graft is of a very specific nature, after a certain amount of physiological differentiation has taken place in the parts transplanted. This physiological differentiation of the cells of the organism has been called "protoplasmic specialization" by Child (1921). In speaking of "self-differentiation" in isolated parts of annelid and mollusk embryos he states (p. 120) " . . . protoplasmic specialization has advanced so far that the regulatory reactions to isolation of parts are limited, and the behavior of the part after isolation is therefore much the same as before." Just when the specialization of the cells of the organ primordium is established in the chick embryo is a matter requiring further investigation, though the results of

the present study indicate that it is at a very early stage. It is conceivable that, for the eye, for example, specialization may occur after the earlier stages used by Danchakoff, for in her experiments, the entire embryo with a part of the blastoderm and not the isolated primordium was transplanted, so that physiological isolation was not complete. The time of such organization differs for different animal classes as has been shown by the experiments on the independent development of isolated blastomeres, some cleavage being determinate from the very first with no post-regeneration. The antero-posterior course of embryonic differentiation in the chick indicates that the time of this specialization varies for different parts of the same embryo.

The results of the study of the organs described indicate that the cells of their parts possess a specificity which is histologically equal to that of the normal. That such a specificity is an innate property of the cells of the embryo at certain stages has, moreover, been shown by numerous investigators by the methods of tissue culture, where the cells behave more or less as independent units. The same principles apply to the development of the subordinate parts of the organs grafted. This is emphasized by the differentiation of the cristæ acusticæ of the membranous labyrinth, the lens and the lenticular zone of the eye, and the various parts of the nasal region and the mesencephalon. The development of a subordinate part when isolated as such, is a problem, the results of which must be established by further investigation. That such parts are capable of differentiation without the stimulation of external factors is proven by their behavior when isolated in the primordium of the organ. No nerve stimulation of the cristæ acusticæ is necessary for their origin, for example, a condition closely resembling that found to exist in the formation of the lateral line sense organs of the amphibian larva by Harrison. The results which have already been obtained indicate that the same general principles are involved in the differentiation of the parts of the other primordia which have been employed in the transplantations.

The limits of development of the isolated parts seem to depend on the mechanical factors involved rather than on the physiological isolation as such. In none of the cases is this more marked

than in those where the otocyst and mesencephalon are used in transplantation. Despite the disorganization produced by the manipulation and by the forces acting on the graft during its growth on the host, the results of the isolation are not marked, differentiation occuring in an approximately normal manner. The absence of the external limiting membrane of the mesencephalon, for example, does not alter the differentiation of the parts as such, but does modify the type of development obtained by the organ as a whole.

Bearing these points in mind, we may say that these results emphasize the mosaic type of the development of the primordia examined, after their origin in the embryo. The time of their origin varies, but once they are physiologically constituted as such, their isolation from the adjacent structures has little effect on the unfolding nature of the realization of the potentialities of their constituent parts. The limits which are imposed on the development of these transplanted organs are dependent on environmental conditions apparently largely of a mechanical nature. The nature of the development of the different cells is apparently very specific, but to what degree, remains the subject for further investigations which are being made at the present time.

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# CYTOLOGICAL CHANGES IN UNFERTILIZED TUBAL EGGS OF THE RAT.

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## INTRODUCTION.

Recent work on artificial parthenogenesis in vertebrates has reopened the question as to the possibility of some degree of parthenogenesis in mammals. Many authors have described division comparable to cleavage in eggs of atretic follicles, but no thorough-going study of the cytological changes in the tubal eggs of mammals has been made until recently. This lack of observation was natural as long as ovulation was thought of as occurring only at copulation, since under these circumstances the possibility that fertilization might have occurred could not have been excluded. Other work indicating that ovulation occurs at regular intervals in several mammals including the rat quite independently of copulation, lead to the question as to how far the cytological changes of the unfertilized tubal egg of the rat might parallel normal development. It was of interest to compare the changes in the unfertilized eggs in atretic follicles, as well as with eggs which have been artifically stimulated to parthenogenetic development. The conditions which have been described in these three cases are so much alike that one is inclined to believe that similar physiological changes are going on in each. Whether these processes are wholly disintegrative in the tubal rat egg; or represent an abortive beginning of parthenogenetic development appears to be a matter of interpretation. Several authors have stated, and this work corroborates the observation, that mammalian eggs disintegrate in the second polar spindle stage if fertilization does not occur soon after ovulation. Many other authors have noted the occurrence of divided eggs, simulating, at least, two, four, or more cell stages, in the lower portion of the oviduct and uterus of mammals. It is the purpose of this paper to trace the cytological changes which occur between ovulation and such apparent cleavage stages.

## Material.

For the material used in the preparation of this paper I am indebted to Drs. J. A. Long and H. M. Evans. I also wish to express my thanks to Dr. Long for helpful encouragement. The material was prepared for a study of ovulation in the rat, *Mus norwegicus*. The females from which the ova were obtained were isolated before parturition and kept from males thereafter, thus preventing the possibility of ova being fertilized. They were killed at different intervals after parturition. The ovaries and oviducts were fixed in Zenker's or in Benda's fluid and stained in carmine, iron hæmatoxylin, or phosphotungstic acid hæmatoxylin. The latter was by far the most useful stain. The Benda material was especially valuable for study of the cytoplasmic elements, but it was less useful than the Zenker preparations for details of nuclear structure, because the darkly staining granules and mitochondria tend to obscure the chromosomes especially in the period of scattering. In the fifty oviducts which were studied eggs were found in the distal portion in twelve cases, in the middle upper portion in ten, in the lower middle portion in seven, and in the proximal portion in nineteen. In all two hundred and fifty-two eggs were examined. As many as nine eggs were found in some oviducts, but three, four, and five were noted much more commonly.

## Observations.

At ovulation the second polar spindle lies parallel to the periphery of the egg with the dyads arranged upon it in typical final prophase position. In any one oviduct all of the ova are at about the same stage. Table I. demonstrates the fact that the commoner stages show a natural sequence with respect to position in the oviduct beginning with distortion of the spindle and scattering of the chromosomes and ending with the unequal fragmentation of a multinucleate cell. It is evident that we are thus provided with a natural seriation of cytological stages of great value in interpretation of the results.

*Stage I.*—It will be noted from Table I. that by far the greatest number of ova found in the distal third of the oviduct were in the second polar spindle stage. The spindle may, however, be slightly turned or even at right angles to its original position

parallel to the periphery. In a few cases some of the dyads have divided and the monads have moved apart from one another on the spindle fibers. First polar bodies are often found, but no evidence of second polar body formation was noted. The cytoplasm shows no marked differentiation, consisting of a rather coarse reticulum enclosing a few large yolk globules and many small granules. Some of them stain deeply and are found in chains. These may be mitochondria. The zona pellucida is

TABLE I.

SUMMARY OF CYTOLOGICAL DATA AS RELATED TO POSITION IN THE OVIDUCT.

| Stage. | Series. | Position in Oviduct.[1] | Number of Eggs. | Cytological Notes. |
|---|---|---|---|---|
| I. | 318 | Distal | 3 | 3 with second spindles slightly rotated in all. |
| | 345 | " | 9 | 2 with second spindles parallel surface, 1 perpendicular; 6 with chromosomes scattered. |
| | 383 | " | 4 | 4 with spindles parallel surface. |
| | 337 | " | 3 | 1 with spindles parallel surface; 1 perpendicular; 1 with chromosomes condensed. |
| | 227 | " | 3 | 2 with spindles parallel surface; slightly rotated in 1. |
| | 10 | " | 4 | 4 with spindles parallel surface. |
| | 103 | " | 7 | Second spindles show some scattering, peripheral cytoplasm vacuolate. |
| | 110 | " | 6 | 4 with spindles parallel surface; 2 show some scattering. |
| | 111 | " | 4 | 4 with spindles parallel surface. |
| | 18 | 2.5 | 2 | 2 with blunt spindles parallel surface. |
| | 33 | 2.5 | 3 | 2 with 2d polar spindles parallel surface; 1 contains 1 large and 1 small spindle. |
| | 113 | 3.5 | 6 | 5 with 2d polar spindles parallel surface; 1 central. |
| | 102 | 3.5 | 1 | 1 with 2d polar spindles parallel surface. |
| | 109 | 3.5 | 3 | 3 with 2d polar spindles parallel surface. |
| II. | 115 | 4 | 9 | 5 with 2d polar spindles parallel surface; fragmentation in others; distortion. |
| | 35 | 4 | 3 | 1 with 2d polar spindles parallel surface; 2 with spindles and vesicles. |
| | 26 | 4.5 | 1 | 1 with chromosomes scattered, vesicles forming about them. |
| | 22 | 4.5 | 2 | 2 with chromosomes scattered. |
| | 367 | 4.5 | 3 | 3 with chromosomes scattered. |
| | 107 | 5 | 3 | 1 with chromosomes scattered; 1 second spindle intact; 1 fragmenting. |
| | 8 | 5 | 3 | 1 with chromosomes scattered, broken spindle; 2 second spindles. |
| | 50 | 5.25 | 1 | 1 with chromosomes scattered, distorted spindle |
| | 114 | 5.5 | 5 | 4 fragmenting, multinucleate; 1 with 3 large vesicles. |
| | 347 | 5.5 | 7 | 6 with chromosomes scattered, 1 with 1 large group; 1 fragmenting. |
| | 4 | 6 | 2 | 2 multinucleate. |

| Stage. | Series. | Position in Oviduct.[1] | Number of Eggs. | Cytological Notes. |
|---|---|---|---|---|
| III. | 325 | 7 | 1 | 1 with 4 equal uninucleate cells. |
| | 308 | 7 | 1 | 1 multinucleate, fragmenting. |
| | 17 | 7.5 | 4 | 4 multinucleate. |
| | 47 | 8.5 | 3 | 3 multinucleate. |
| | 403 | 8.5 | 9 | 9 multinucleate, lobing irrespective of nucleus, fragmenting. |
| | 12 | 8.5 | 3 | 2 multinucleate; 1 with spindle parallel surface. |
| | 354 | 8.5 | 5 | 3 multinucleate and fragmenting; 2 with scattered chromosomes. |
| | 42 | 8.5 | 2 | 2 multinucleate, fragmenting irregularly. |
| | 453 | 8.5 | 9 | 9 fragmenting, with one or more nuclei; irregular. |
| | 452 | 9 | 1 | 1 multinucleate. |
| | 37 | 9 | 5 | 5 fragmenting with unusual regularity, 2 4 cells. |
| | 344 | 9 | ? | Masses of uni- or multinucleate fragments. |
| | 324 | 9 | 3 | 3 zona broken, fragments disintegrating. |
| | 388 | 9 | 2 | 1 uninucleate cell; 1 group of 4 equal cells in a single row. |
| | 48 | 9 | 1 | 1 with 4 multinucleate cells. |
| | 32 | 9 | 5 | 5 multinucleate. |
| | | | 152 | |

[1] The figures indicate the distance of the eggs from the oviduct; thus stage 1. includes all eggs in the upper part of the oviduct, *i.e.*, from 1–3.5, while 9*c* is the region nearest the uterus.

| Position in Oviduct. | Normal Maturation Spindle Parallel Surface | Normal Maturation Spindle Slightly Retarded | Normal Maturation Spindle Perpendicular | Scattered Chromosomes. | Unfragmented Multinucleate Cells. | Fragmenting Multinucleate Cells. | Fragmented Eggs. | Two Spindles in One Egg. | Central Chromosomes Condensed. | 2, 4 Cell Stages. |
|---|---|---|---|---|---|---|---|---|---|---|
| 1–3.5..... | 34 | 4 | 2 | 15 | 0 | 0 | 0 | 0 | 2 | |
| 4–5..... | 6 | 0 | 0 | 16 | 2 | 1 | 0 | 1 | 0 | |
| 5–8.5..... | 1 | 0 | 0 | 7 | 14 | 13 | 0 | 1 | 0 | 1 |
| 8.5–9*c*... | 0 | 0 | 0 | 2 | 0 | 6 | 22 | 0 | 0 | 2 |
| Totals... | 41 | 4 | 2 | 40 | 16 | 20 | 22 | 2 | 2 | 3 |

evident in all of the Benda and in some of the Zenker preparations as a wide homogeneous layer. Why it should have been preserved in some of the Zenker preparations and dissolved off in others is not understood. The follicle cells appear to be quite normal.

All of the eggs in twelve oviducts contained spindles either parallel to the periphery or somewhat perpendicular to it. In all, 47 eggs were found to be in this stage and of these forty were in the upper half of the oviduct.

*Stage II.*—In the upper middle portion of the oviduct the spindle is beginning to break down and the chromosomes are becoming scattered. In most of the unfertilized eggs studied the second maturation spindle remains parallel to the surface of the egg and there breaks down, the fibers splitting off and carrying the dumb-bell shaped chromosomes with them. This process varies in completeness in different ova. The spindle may be evident after most of the chromosomes are vesiculate, or it may disappear almost completely as the chromosomes scatter.

Eggs were found in the upper middle portion of the oviduct in ten cases, and in all forty eggs had broken spindles and scattering chromosomes. Thirty-one of these were in the upper half of the oviduct. The breaking down of the spindle results, at times, in a simulation of multipolar spindles but their irregularity and the fact that the chromosomes are usually widely scattered indicate that they result from the breaking down of a single spindle. Sometimes the spindle, when quite degenerate, is no longer fibrillar but appears as a dense homogeneous mass of material of a very distorted shape.

In two oviducts eggs were found in which the spindle was central, and the cytoplasm about it unusually dense. The spindle appeared shrunken and the chromosomes had coalesced into a compact mass. Such eggs were also found well down in the oviduct.

*Stage III.*—The vesicle formation which began in Stage II. proceeds until multinucleate cells are formed. A liquid gathers about a single chromosome or a group of them. The chromosomes then swell and break up into large and finally into small granules so that the nuclei assume the typical resting condition. They vary greatly in size and number, different cells in the same oviduct containing from one to ten nuclei which differ greatly in size. The appearance of the nuclei differs with the fixative used. In eggs fixed in Benda the chromatin lines the inside of a clear vesicle, most of it being in a mass on one side. In eggs fixed in Zenker the chromatin appears as granules on a net. Central spindles in different stages of degeneration were found in three oviducts. In one of these one egg contained a central spindle while the other three resembled typical resting cells. In the

other two, five eggs contained degenerating spindles while the rest were in an advanced stage of fragmentation.

Cracks often appear in the cytoplasm but the zona is still usually intact at this stage. The follicle cells are often grouped into small syncytia containing many small crescent shaped nuclei.

The eggs of stage three were mostly found in the lower middle portion of the oviduct. In all thirty-six ova were multinucleate. Of these sixteen were unfragmented, and twenty were beginning to fragment.

*Stage IV.*—Fragmentation begins almost simultaneously with vesicle formation but is usually completed in the most proximal portion of the oviduct. The fragments vary in number from two or three to many. They may be approximately equal in size or very unequal. A fragment may contain one to several nuclei or none. Fragmentation occurs quite independently of the vesicles when many are present. It may take place either by a process of lobing, or, more commonly, by the appearance of cracks in the cytoplasm.

Some of the ova in the lower portion of the oviduct resemble 2, 3, and 4 cell stages very closely. Three two, one three, and two four cell stages were found. In oviduct 32 only one egg in five resembled a normal condition, the rest being multinucleate and more or less fragmented. In oviduct 37 two two and one four cell stage were found, while two eggs were multinucleate and fragmented. Number 325 contained very degenerate fragments and one four cell stage. The cytoplasm is very degenerate in most of the fragmenting eggs. A zona may or may not be present but if it appears at all it is nearly always ruptured. The fragments are usually quite devoid of covering far down in the tube, and it is impossible to tell how many ova they come from. The follicle cells are now empty shells devoid of protoplasm.

Complete fragmentation was the rule in nineteen oviducts. All of these were found in the lower third of the tube. In all twenty-two ova had reached this stage but this number does not represent the total since in several cases degeneration had proceeded so far that the figures for them had to be omitted.

## Literature and Discussion.

The observations upon atretic and unfertilized tubal eggs of mammals agree essentially but the interpretations are rather various especially with regard to atretic eggs. This is easily comprehensible since no natural method of seriation is provided in atretic material. On the other hand one can easily determine the position of tubal eggs in the oviduct, and as these data show, can then demonstrate that such eggs pass thru a regular series of changes as they descend the duct.

In eggs which are liberated into the fallopian tube the second maturation spindle sometimes rotates to a position at right angles to the surface. The dyads also divide occasionally so that the second anaphase really appears to begin in some unfertilized eggs. In by far the greatest number of eggs in the upper third of the oviduct, however, the second polar spindle is parallel to the periphery and intact, with the dyads still in late prophase position. The spindle may or may not be somewhat broken. No cytoplasmic changes were noted at this stage. In eggs in two oviducts the spindle was central in position.

Three of the facts cited above might be interpreted as indicating that the cell processes may not all be wholly degenerative at this stage. Firstly, the spindle may rotate as if in preparation for the second polar division. Secondly, some of the dyads may divide, and thirdly, the spindle may be found in the center of the egg. Eggs with central spindles all showed one of two degenerative conditions; the chromatin simply clumping into a single mass and the cytoplasm shrinking and becoming denser, or in other cases the cell wall may break and the very vacuolate cytoplasmic contents scatter. There is no indication in the tubal rat material that normal cleavage ever occurs in these eggs altho it may of course be possible that more material might include such stages since a few eggs give the appearance of approximately normal early cleavage stages. In by far the greatest number of eggs degeneration begins with the failure of the second polar division. It is of some interest that the formation of but one polar body is a characteristic feature of development of most naturally parthenogenetic animal eggs.

Degeneration has been shown to begin with the breaking of the

spindle and the consequent scattering of the chromosomes throughout the cytoplasm. Kingery ('13) described the same type of degeneration for atretic eggs of the mouse. The cytoplasm and chromosomes interact so that the latter at first appear to be surrounded by a clear liquid. They then swell and form little vesicles resembling nuclei. Where several chromosomes are contiguous large vesicles result. Charleton ('17) described the nucleus of tubal mouse eggs as forming from the entire chromosome group, which then gives rise to a multinucleate cell by lobing. The seriation here described demonstrates that this is not the case in the rat. If several contiguous nuclei were fusing, pictures such as Charleton has described as due to lobing would be obtained. Wilson ('01) has described syncytia in artificially parthenogenetic Toxopneustes eggs as resulting from a failure of cytokinesis following nuclear division by a mitotic process. The common appearance of broken spindles and scattered chromatin in the upper part of the oviduct as well as the difference in size of the vesicles indicates strongly that this is not the case in tubal rat eggs.

No true multipolar spindles, monasters or cytasters such as were described by Henneguy ('04, '05) for atretic rat eggs, by Newman ('13) for atretic eggs of the armadillo, and in artificially parthenogenetic Toxopneustes eggs by Wilson ('01) were found. One egg showed two partial spindles at opposite poles of the cell. Lams ('13) interpreted a similar condition in a tubal egg of the guinea-pig as indicating that the first polar body had not been detached from the egg, but notes that a similar case could not be so interpreted since the first polar body was clearly present. In view of the fact that the second polar spindle of tubal rat eggs sometimes snaps in two after great elongation and distortion it seems best to consider such pictures as originating in this manner. During the scattering of the chromosomes the cytoplasm becomes more coarsely granular and the meshes more open. Wilson ('01) described the cytoplasm of *living* artificially parthenogenetic eggs as becoming coarser by clumping of microsomes preceding aster formation.

One cell was found with one, and one with two resting nuclei. They are probably the result of chance grouping of chromatin

into one or two rather than into many masses.[1] When few nuclei are present they tend to occupy the center of the egg. Eggs containing resting nuclei were lower in the oviduct than any of the eggs containing spindles, hence it is probable that spindles never form following the disintegration of the second polar spindle in tubal rat eggs. Newman ('13) interprets his atretic armadillo material as indicating that new mitotic figures with asters form following a return to the resting condition after the failure of the second polar division, but none of the rat eggs with central spindles showed any indication of astral radiations.

There is no indication in the rat material that fertilization by a polar body ever occurs. The first polar body is completely separated from the egg before ovulation takes place.

Fragmentation occurs almost independently of the newly formed "nuclei," so that some of the resulting cells contain nuclei and some do not. Newman ('13) and Charleton ('17) describe a similar cutting off of anucleate portions of the egg and consider it as a form of deutoplasmolysis. Newman believes that such a process would be advantageous to a cell system governed by a haploid nucleus. Since in the rat eggs the distribution as well as number and size of the "nuclei" is haphazard in the fragments it seems improbable that the process should be considered normal. In the tubal rat material it appears that the fragmentation process is less abnormal when few nuclei are present in the egg, and that it may at times be fairly normal is indicated by the fact that a few normal-looking 2, 3, and 4 cell stages were found. In his observations on unfertilized eggs of Toxopneustes Wilson ('01) states that most of the blastomeres resulting from a simultaneous cleavage of syncytia contain nuclei but that some do not. Here also the fewer nuclei present, the more normal the division. Thus in tubal rat eggs as in artificially parthenogenetic ones, a few may have more vitality than the rest, and these may possibly be considered as possessing a very limited parthenogenetic capacity.

In the lowest portion of the oviduct all of the ova were obvious-

[1] The possibility that sperm may live in the oviduct for several days and then be capable of fertilization should, however, be taken into consideration in interpreting these occasional normal looking cases. Wilson ('01) also found some unfertilized eggs which resembled fertilization stages.

ly degenerate. They might be expected to enter the uterus, if at all, as mere shells and scattered cytoplasm. The zona is either broken or absent at this stage.

## Conclusion.

Following ovulation most of the changes undergone by tubal rat eggs are distinctly abnormal. The central position of the spindle, the occurrence of three uni-, and one binucleate cell, the coarsening of cytoplasmic structure, the observation that division is more or less regular according to the number of "nuclei" which are present, and the discovery of a few two, three and four cell stages may be thought to indicate some tendency towards normal development in certain of the unfertilized eggs. On the contrary, the fact that the central spindle is rare and always degenerate, that only three uninucleate cells were found, the great preponderance of multinucleate cells, and the fact that only very degenerate fragments are found near the uterus, make it necessary to conclude that although certain auto-regulative processes may occur in a few ova, a definite type of degeneration is the rule.

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